Electron Microscopic Diagnosis of Human Flavivirus Encephalitis
Use of Confocal Microscopy as an Aid

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The distinction between intracranial viral infections and inflammatory conditions requiring immunosuppression is important. Although specific laboratory reagents are readily available for some viruses, diagnosis of arbovirus infection is more difficult. Transmission electron microscopy (TEM) theoretically allows identification of viral particles independent of reagent availability, but it has limited sensitivity. We report two cases of human flavivirus encephalitis diagnosed by TEM. Laser scanning confocal microscopy (LSCM) was used in one case to survey unembedded tissue slices for focal abnormalities, from which fragments smaller than 1 mm² were excised for epoxy embedding. This facilitated TEM identification of intracytoplasmic budding, 35–40 nm spherical virus particles, confirmed by serology as St. Louis encephalitis. In contrast to mosquitoes and newborn mice, in which high viral loads are associated with minimal tissue responses, these biopsies showed florid angioedemotic inflammation and microgliosis, with rare virions in necrotic perivascular cells and astrocytes. To our knowledge, this represents the first ultrastructural study of St. Louis encephalitis in humans, indicating the potential value of LSCM-aided TEM.

Key Words: Flavivirus—Ultrastructure—Diagnosis—Electron microscopy—Confocal microscopy.


Mosquito-borne viral encephalitides are a group of relatively uncommon, potentially fatal diseases that are highly significant from a public health perspective. Within the United States, eastern and western equine encephalitis (alphavirus), St. Louis encephalitis (flavivirus), and the California encephalitis serogroup, including La Crosse encephalitis (bunyavirus), are the most significant.

Currently, diagnosis is based on detection of IgM antibodies or comparison of acute and convalescent sera. The sporadic nature of disease outbreaks often results in a low clinical index of suspicion, which is compounded by the wide variety of potential viruses involved and the lack of readily available diagnostic reagents. Diagnoses are often not confirmed until convalescence or death. In some instances, clinical diagnoses of primary central nervous system (CNS) vasculitis, lymphoma, or other inflammatory conditions requiring immunosuppression are entertained and make earlier detection of arboviral infections desirable.

Transmission electron microscopy (TEM) has been central in elucidating the structure and classification of viral pathogens, but its diagnostic usefulness for patient treatment has been limited by the cost to sampling efficiency ratio imposed by the small volume of tissue that can be examined. We found only two reports of eastern equine encephalitis that included direct ultrastructural identification of the alphavirus, one autopsy study of human tick-borne flavivirus, and no TEM studies of human St. Louis encephalitis in the literature. This may reflect the low viral titers typically achieved within dead-end hosts, such as humans and horses. Although viral antigens have been identified by immunofluorescence in rapidly fatal human cases, St. Louis encephalitis virus levels are often below the detection threshold for immunofluorescent reagents. Advances of TEM include visual confirmation of active infection by clinically unsuspected or unusual viruses and information concerning pathophysiologic mechanisms of infection and spread.
Laser scanning confocal microscopy (LSCM) can be used to survey thick, unembedded sections of tissue for evidence of viral cytopathic changes (see reference 3 for review). These select areas then can be processed for ultrastructural examination, eliminating the need to examine large numbers of TEM blocks randomly selected during gross tissue evaluation. This confocal microscopy technique has been used previously for ultrastructural examination of several inclusion-forming DNA viruses by using propidium iodide nuclear stains or immunofluorescent reagents against specific viral antigens. In addition to identifying viral cytopathic changes, nuclear-staining patterns can be used to highlight patchy areas of hypercellularity or inflammation and focal regions of tumor rosette formation or viability. By allowing histologic assessments of thick, unembedded tissue slices, LSCM prescreening thus may enhance the overall effectiveness of TEM as a diagnostic modality.

We report two cases in which TEM visualization of flavivirus particles in brain biopsies was central to clinical management of the patients. The first biopsy, from a patient with Wiscott-Aldrich syndrome, was characterized by diffuse encephalitis from which randomly selected TEM blocks were positive for virions. In the second case, LSCM-aided block selection was crucial for the ultrastructural identification of budding virions within a focal area of histologic abnormality. In conjunction with a single serologic study showing titers for St. Louis encephalitis, TEM evidence of active flavivirus infection resulted in immediate cessation of aggressive immunosuppressive therapy that had been initiated for presumed vasculitis.

METHODS

Initial Tissue Handling

Biopsy material was obtained fresh from the operating room by the neuropathologist on call for intraoperative consultations. After gross inspection, most of the tissue, including leptomeninges, was submitted for routine paraffin histology and a portion was retained for possible TEM studies (Fig. 1). For routine TEM submission, tissue was finely minced and placed directly in glutaraldehyde. For possible LSCM-aided TEM, a perpendicular slice of tissue including leptomeninges, cortex, and superficial white matter, approximately 2 mm thick and 1 to 2 cm² in area, was retained in buffered formalin.

LSCM Selection of Blocks

The formalin-fixed slices were attached to chucks with fast-drying cyanoacrylate glue. Using a vibrating microtome, 100- to 200-µm thick sections were cut from the unembedded tissue. After glutaraldehyde postfixation, the sections were stained for 10 to 15 seconds with propidium iodide and placed on a slide under a saline-mounted glass coverslip, as previously described. Tissue stained in this manner shows bright yellow-white nuclear fluorescence as a result of propidium iodide binding to nuclear DNA, with less intense cytoplasmic fluorescence because of RNA binding or glutaraldehyde-induced autofluorescence. A slit-scanning Meridian InSIGHT Plus confocal microscope (Genomic Solutions, Inc., Ann Arbor, MI, U.S.A.) was used to identify areas of vasculitis or perivascular inflammation, microglial nodules, and neuronal necrosis or gliosis using a green excitation filter cube. The area of interest was marked using an inked attachment (Nikon, Melville, NY) that is mounted in place of an objective. A diagram showing relative landmarks was drawn. With a dissecting microscope, excess tissue was trimmed away to form an asymmetric polygon so that the tissue could be oriented for sectioning from the same face that had been examined. The fragments were then reexamined by LSCM to ensure that the area of interest had not been lost. In this way, select areas of appropriate size for TEM processing (<1 mm²) were generated.

Processing of TEM Blocks

Tissue fragments that were conventionally (randomly) selected or selected with the aid of LSCM were washed, stained with 1% osmium followed by 1% uranyl acetate, infiltrated with Spurr's resin (Electron Microscopy Sciences, Fort Washington, PA), and sectioned as previously described. Thin sections were poststained sequentially with uranyl acetate and lead citrate and examined with a Philips EM300 electron microscope (FEI/Philips, Hillsboro, OR, U.S.A.).

CASE REPORTS

Case 1: Atypical Lymphocytic Encephalitis Associated With Flavivirus Infection

Clinical History

A 19-year-old man with a medical history of Wiscott-Aldrich syndrome and febrile seizures was referred to be examined for new, recurrent seizures and abnormal results from a brain magnetic resonance imaging (MRI) study. Seizures were of multiple types, including right-sided focal motor seizures with secondary generalization, vision loss followed by secondary generalization, and generalized tonic-clonic seizures. Postictally, he was confused and aggressive. During a 6-month period, the patient developed dysarthria, aphasia, ataxia, and low-grade fevers. A biopsy specimen was obtained from an enlarged lymph node but was negative for lymphoma or a specific infectious etiology.
Brain MRI showed multifocal lesions, including several that enhanced with contrast (Fig. 2). Bilateral occipital lobes, right frontoparietal lobe, right thalamus, left corona radiata, and the pons were involved most prominently. Compared with an MRI taken 3 months earlier, several lesions had enlarged and one had regressed. Lumbar puncture and cerebral arteriogram did not suggest specific diagnosis.

The patient’s temperature was 38°C, and other vital signs were normal. He had a diffuse papular rash and mild cervical adenopathy. He was alert but had a nonfluent aphasia. Results of cranial nerve, motor, and reflex examination were normal. His gait was ataxic with slowed limb movements. Chest radiograph findings were normal. Blood cultures grew Streptococcus pneumoniae, which was treated with amoxicillin. Echocardiography
was negative for endocardial vegetations. Because of concern for possible lymphoma, the patient underwent a right frontoparietal brain biopsy.

Pathologic Findings

Examination of the 2.3 \times 1 \times 1 \text{ cm} biopsy specimen showed multiple extensive areas of abnormality. Atypical, dense, angiocentric lymphoid infiltrates disrupted the white matter with extension into adjacent astrogliotic cortex. The infiltrate consisted of lymphocytes with atypical convoluted nuclei, histiocytes, eosinophils, occasional neutrophils, and occasional plasma cells. Angiodestructive transmural involvement of small to medium-sized vessels was associated with partial occlusion and occasional thrombosis (Fig. 3A). Scattered neurons undergoing neuronophagia were identified (Fig. 3B). Histochemical stains for fungi, bacteria, and mycobacteria and immunostains for herpesvirus antigens were negative. Immunophenotyping of the lymphocytic infiltrate showed mixed reactivity with T lymphocyte predominance. Examination of two conventionally prepared TEM grids showed numerous small (35–40 nm) viral particles within cytoplasmic cisternae, probably endoplasmic reticulum (Fig. 3C). Some of these virions were arranged in paracrystalline arrays, and occasional virions budding through cytoplasmic membranes were identified (Fig. 3D). The virions were morphologically most consistent with flavivirus particles. Active viral infection, including a larger paracrystalline array, was identified in astrocytic cells containing bundles of intermediate filaments (Figs. 3E, F). Other infected cell types were not identified definitively, although some infected cells appeared to be adjacent to basal lamina material.

Hospital Course

The patient was discharged and lost to follow-up. Nearly a year later, he died of unspecified cause after an admission to the emergency department at another institution.
FIG. 3. Case 1. (A) Light micrograph showing perivascular inflammation involving parenchymal vessel. Arrow, residual lumen of vessel. (B) Light micrograph showing neuronophagia. Arrowhead, neuron; arrows, inflammatory cells. (C) Low-magnification electron micrograph showing small, scattered virions throughout cytoplasm (arrowheads). A cluster of virions (arrow) is enlarged in panel D. (D) High-magnification electron micrograph of virions from panel C. The virions have envelopes that are not clearly visible in the micrograph; many of the virions are partially or totally surrounded by associated cell membranes. One of the particles (arrow), in the process of budding from a portion of cell membrane, is shown at higher magnification in the inset. (E) Low-magnification electron micrograph showing infected cell processes within the neuropil. Note the large cluster of flavivirus particles within one of the denser processes (arrow). This is shown at high magnification in panel F. The nuclei of two cell bodies in the vicinity are included for orientation (bottom corners). (F) High-magnification electron micrograph of the infected cell shown in panel E. The cluster of virions is surrounded by bundles of intermediate filaments, a feature typical of astrocytes. Bars in A and B = 10 μm; bars in C and E = 1 μm; and bars in D and F = 100 nm.
Case 2: St. Louis (Meningo)encephalitis With Secondary Vasculitis

Clinical History

A 34-year-old, previously healthy man had a 2-week history of bilateral throbbing headache, nausea, and fever. He was from New York City but had been visiting North Carolina for several weeks. He had a generalized tonic-clonic seizure followed by worsening headache and anorexia. Results of noncontrasted computerized tomography of the brain was normal, and cerebrospinal fluid (CSF) analysis showed 68 white blood cells/mm³ (36% neutrophils, 50% lymphocytes, and 13% monocytes), 62 mg/dL protein, 60 mg/dL glucose, and negative cultures. He was admitted after a second seizure 2 days later.

On admission, results of general and neurologic examinations were normal. Brain MRI showed abnormal leptomegeal enhancement in the posterior right temporal lobe and Sylvian fissure overlying areas of T2 signal abnormality (Fig. 4). Results of HIV testing, tests for tuberculosis and syphilis, rheumatologic studies, chest radiograph, and multiple blood cultures were negative. Despite this treatment, he had recurrent seizures and continued headache and developed fever and confusion during the next 2 weeks. Repeat CSF examination showed 188 white blood cells/mm³ (19% neutrophils, 70% lymphocytes, and 10% monocytes) and 112 mg/dL protein. MRI showed progression of gyral enhancement and new areas of signal abnormality in the right medial temporal lobe. The patient underwent brain and meningeal biopsy based on his worsening course, CSF studies, and MRI.

Pathologic Findings

Serial step sections of the 1.3 × 1.2 × 0.3 cm specimen showed a single circumscribed abnormality (Fig. 5A). In this region, small meningeal and superficial penetrating vessels were involved by an intense, transmural inflammatory infiltrate consisting of lymphocytes and neutrophils with scattered histiocytes (Fig. 5B). Fibrinoid vessel necrosis was apparent, as were red neurons, gliosis, and neuronophagocytosis. A larger meningeal artery was uninvolved. No viral inclusions were apparent. No organisms were identified with histochemical stains for bacteria, fungi, and mycobacteria or with immunohistochemical stains for herpesviruses. Examination of nine conventionally processed grids by TEM also showed no evidence of viral particles, gliosis, or inflammation.

Several fragments of brain measuring up to 7 × 5 × 5 mm then were surveyed by LSCM, with selection of three blocks for TEM. No virus was identified in two areas of necrosis or microgliosis (Figs. 5C, D). Rare virions were identified in poorly preserved, degenerating cells adjacent to an inflamed meningeal vessel that had been located by LSCM (Fig. 6). These round, 40-nm particles were associated with abundant intracytoplasmic membranes through which rare virions appeared to be budding (Fig. 6D). The visualization of budding flavivirus particles, in conjunction with IgG titers, established the diagnosis of St. Louis encephalitis.

Hospital Course

Postoperatively, the patient was initially treated with high-dose steroids for the preliminary diagnosis of cerebral vasculitis and parenchymal ischemia. He improved, and cyclophosphamide was added after special stains for

FIG. 4. Case 2. (A) T1-weighted MRI scan of the brain after administration of gadolinium shows leptomeningeal enhancement of the temporal cortex associated with gyral edema and an enhancing insular cortex lesion. (B) The corresponding area shows a bright signal on a T2-weighted MRI scan.
organisms and conventional TEM results were negative. Shortly after discharge, serology for St. Louis encephalitis virus returned weakly positive (IgG 1:80, IgM <1:10). The definitive identification of budding flavivirus particles by LSCM-aided TEM resulted in immediate cessation of cyclophosphamide and rapid tapering of steroids, circumventing the need to wait several weeks for confirmatory serology findings. The patient recovered and remained neurologically healthy 1 year after discharge.

**DISCUSSION**

We have presented two cases in which active flavivirus infection was visualized in human biopsy material by TEM. Of the various mosquito- and tick-borne flaviviruses, St. Louis encephalitis was favored by its geographic distribution in North America, and this diagnosis was confirmed serologically in one case. Previous ultrastructural descriptions of the morphology and topography of St. Louis encephalitis virions were limited to experimental work in mosquito vectors and newborn mice,15,18 most likely because of the greatly diminished viral load observed in weanling and adult mammals.1,14 The sampling problem inherent to diagnostic TEM is accentuated further by the low virus levels and patchy nature of flavivirus infection in immunocompetent hosts. The use of LSCM to select regions for subsequent ultrastructural analysis allowed for improved sampling of the focal lesion observed in the second case.

In mosquito salivary gland epithelium and the brains of infected newborn mice, St. Louis encephalitis is a

**FIG. 5.** Case 2. (A) Low-magnification light micrograph showing a focus of intense meningeal perivascular inflammation (arrows). The brain is hypercellular in the region immediately surrounding this focus. Aside from this meningeal and superficial cortical focus, the remainder of the biopsy specimen was histologically normal. (B) Light micrograph showing mixed perivascular inflammation involving parenchymal vessel. Although the infiltrate consists mainly of mononuclear cells, scattered neutrophils (arrow) can be identified adjacent to the vessel lumen. Neuronophagia was also evident (arrowheads). (C) Light micrograph showing glial nodule in the cortex. (D) Confocal micrograph showing a glial nodule identified by propidium iodide nuclear stain. At higher magnification (inset), a neutrophil nucleus is visible (arrowhead). Bar in A = 1 mm; bars in B-D = 10 μm.
characteristically round, 37- to 38-μm virion with a 27-μm dense core and closely apposed lucid envelope.\textsuperscript{15,18}

The viral particles bud from the cytoplasm into the endoplasmic reticulum, Golgi apparatus, or perinuclear envelope, and infected cells are characterized by an intense proliferation of intracytoplasmic membranes. At higher densities, the virions are packed frequently in ordered paracrystalline arrays. Suckling mice typically develop fulminant, overwhelming infections with perivascular edema and neuronal necrosis, but little evidence of a host response.\textsuperscript{15}

In contrast, human infections are histopathologically characterized by microgliosis, neuronophagia, and abundant parenchymal, perivascular, or leptomeningeal inflammatory infiltrates.\textsuperscript{6,9} Although neutrophils typically are not included in the histologic descriptions of St. Louis encephalitis coming to autopsy, the biopsy specimens of the second case, taken 1 month after initial presentation, showed fibrinoid vessel necrosis with a mixed lymphocytic and neutrophilic infiltrate. Thus, neutrophils may be a more prominent early component, in accord with the serial CSF studies of this patient. Although neurons appear to be the primary target in newborn mice, both human cases showed clear infection of astrocytes and perivascular cells, without definitive identification of infected neurons. Ultrastructural studies of the related eastern equine encephalitis also indicate that the immune state of the host may affect not only histopathologic parameters, but also the cellular and subcellular distribution of viral particles.\textsuperscript{2,11,16}

Immune immaturity is associated with rapid progression, high mortality rates, diffuse distributions of lesions, and high viral load within the brain, which facilitate identification of viruses. Likewise, fulminant human cases characterized by death within 1 to 2 weeks and still-negative serologies showed sufficient viral burdens
for detection by immunofluorescence microscopy, whereas fatal cases showing evidence of a systemic immune response during life often did not. It is interesting to note that our first patient, in whom viral particles were detected by conventional TEM block selection, suffered from a partial immunodeficiency. There was a sufficient number of viruses in several cells to form small, ordered paracrystalline arrays. The biopsy specimen from this patient with Wiscott-Aldrich syndrome showed diffuse inflammatory changes with numerous atypical lymphocytes, in contrast to the focal lesion of the second patient, who required LSCM-aided block selection for identification of rare virions.

Because of the epidemic potential for arbovirus infections, early identification of mosquito-borne viruses is essential for public health measures. For some viruses, negative staining is an alternative technique to thin sectioning that can be used to identify virions in fluids such as CSF and occasionally extracts of homogenized tissue. The complete virion or nucleocapsid, however, must have distinct morphology. In contrast, flaviviruses and most other arboviruses would be unrecognizable by negative staining because they are small enveloped viruses that do not have distinct external or internal characteristics. Thus, TEM of thin sections, as performed in these two cases, would be necessary. In one county that initiated active surveillance for arboviruses, thin section TEM was used for rapid presumptive identification of pathogenic viruses based on structure and subcellular location, followed by confirmation using directed serologic tests. In addition to an earlier institution of public health measures, such as controlling mosquito infestation, early identification of arbovirus encephalitis may be critical to the treatment of individual patients, particularly if diseases requiring immunosuppression are included in the differential diagnosis.

Because the seroprevalence of St. Louis encephalitis is nearly 4% in some areas of the eastern United States, an isolated IgG titer is difficult to interpret. Depending on the clinical situation, however, confirmation by sequential serologies may take too long. Because primary CNS vasculitis carries a significant risk of mortality unless treated rapidly with aggressive immunosuppression, our second patient had been administered cyclophosphamide therapy after conventional pathologic studies failed to reveal organisms. The early detection of a productive flavivirus infection by electron microscopy was important to the clinical treatment of this young man.

Although patients with primary CNS vasculitis often show leptomeningeal enhancement and abnormal cerebral angiogram findings, the clinical and radiologic findings are neither sensitive nor specific for vasculitis. Because primary angiitis of the CNS is primarily a diagnosis of exclusion, an unknown number of primary vasculitis cases actually may represent responses to undetected viruses. Varicella zoster DNA was recently discovered during reanalysis of a classic case of vasculitis that had been originally presented in the New England Journal of Medicine. Although neuronophagia and microglial nodules suggest infectious etiologies, only leptomeningeal and perivascular lymphocytic infiltrates may be present at the edge of the lesion, and these lymphocytic infiltrates may be sufficiently atypical to suggest lymphoma. Thus, the possibility of infection must be considered in the histopathologic differential diagnosis for primary CNS vasculitis and lymphoproliferative disorders.

Close communication between clinician and neuropathologist is necessary for optimal management of brain biopsies. As illustrated in Fig. 1, intraoperative notification of the neuropathologist concerning a biopsy specimen in which vasculitis or infection is suspected allows the tissue to be properly triaged. For evaluation of vasculitis, infection, or noninflammatory vasculopathies, histologic examination of leptomeningeal and intraparenchymal vessels is necessary, as is distinguishing between ischemic and encephalitic changes. In some instances, the tissue of the biopsy specimen may be insufficient for retaining a slice in formalin, particularly when a variety of other ancillary studies, such as culture, molecular diagnostics, and immunofluorescence, is desired. Although focal regions of tissue can be dissected from paraffin blocks or thawed directly into glutaraldehyde for subsequent electron microscopy studies, preservation is poor and likely limits diagnoses to the larger, inclusion-forming DNA viruses, such as JC polyoma virus and herpesviruses. Histologic assessment of the focality or diffuseness of inflammatory changes can be used as a guide to determine whether LSCM may aid in maximizing TEM sampling of infected areas. Communication between clinician and pathologist concerning the clinical differential diagnosis and appropriate serologic studies enhances the value of information derived from brain biopsies.

To summarize, early identification of arboviral infections is important at the individual and public health levels. Although more common viral pathogens, such as herpesviruses, can be identified rapidly by immunofluorescence or immunohistochemistry, diagnostic reagents for arboviruses are not widely available. Ultrastructural identification of replicating viral particles in surgical biopsy material can be critical for patient treatment, particularly in conjunction with serologic methods. With the diffuse or focal nature of the histopathologic changes as a guide, random or LSCM-aided block selection can be used. LSCM survey methods also may be useful for other diagnostic ultrastructural or molecular pathology applications.

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REFERENCES