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LEARNING OBJECTIVES

On completion of this exercise, the participant should be able to

- recognize various clinical manifestations of herpes simplex virus (HSV).
- recognize various gross and microscopic manifestations of HSV at autopsy.
- determine the most appropriate method for the definitive diagnosis of HSV from autopsy samples in various autopsy circumstances.
- implement various ancillary testing methods with due attention to sample preservation technique.

HISTORY

At a routine 1-week checkup, a primary care physician found his 7-day-old male patient to be cyanotic and hypothermic, with a rectal temperature of 94°F. The boy was promptly transported to an emergency department, where his temperature was measured at 91°F (32.7°C). He was admitted to an intensive care unit; by this point the child had become lethargic and severely jaundiced. Echocardiography revealed no coarctation of the aorta. Lumbar puncture was easily performed, but during the procedure the child became apneic and bradycardic. He was emergently ventilated, eventually intubated, and placed on a ventilator. Cerebrospinal fluid (CSF) analysis revealed an elevated protein level, low glucose concentration, 1 white blood cell, and 1 red blood cell. Blood chemistry studies revealed glucose levels of less than 20 mg/dL (reference range, > 45 mg/dL), after aggressive administration of 12.5% dextrose in water.[1] Laboratory tests did not reveal any other obvious underlying abnormalities.

The patient's condition deteriorated and he experienced cardiac and respiratory arrest several times, responding positively to epinephrine and dopamine doses on each occasion. Bicarbonate was administered repeatedly to bolster flagging levels, although the patient's concentration never rose above 10 mmol/L (reference range, 15-18 mmol/L).[2] The patient's potassium concentration was elevated above 9 mEq/L (reference range, <6.0 mEq/L), and his calcium level was low.[3] The patient's laboratory results indicated that he was in disseminated intravascular coagulopathy, and hemostasis was therefore difficult to attain. Multiple needle and lancet punctures for blood collection caused significant anemia. Attempts to control blood loss through blood product administration were unsuccessful. The patient's hemoglobin concentration was less than 6 g/dL (normal at 7 days of age, 17.9 g/dL) when last measured.[4] The patient became bradycardic and asystolic, and he died after a last attempt at resuscitation 9 hours after initial presentation. A definitive explanation for the infant's severe debilitation was never determined clinically. An autopsy was requested.

The mother was 19 years old, gravida 1 para 1. Her pregnancy and delivery were uncomplicated. The only significant medical history was that the mother was a smoker.

At autopsy, the myocardium and lungs were discolored yellow-tan, and the skeletal muscle was pale. The liver was covered with a smooth, glistening capsule. The liver parenchyma was dark red-brown and vaguely nodular.

Microscopic examination of the liver revealed hemorrhage, necrosis, and predominately mononuclear inflammation, along with the cytologic features shown in **Images 1** and **2.** The lungs appeared relatively bloodless. Microscopically, the lungs demonstrated no obvious cytologic abnormalities, but exhibited widespread interstitial pneumonitis (**Image 3**), as well as focal areas of hemorrhage, diffuse alveolar damage, microthrombi, and necrosis. The thymus contained foci of extravasated blood.

Postmortem ancillary studies were performed. A serum drug screen was negative. A lung culture was positive for a few *Enterobacter cloacae* and was positive for herpes simplex virus (HSV). Blood culture was positive for *Staphylococcus aureus*. The CSF culture was negative. A metabolic profile for inborn errors of metabolism was also negative.

The cause of death was determined to be HSV infection, and the manner of death was natural.

AUTOPSY DIAGNOSIS OF NEONATAL HERPES SIMPLEX VIRUS

For a review of the clinical aspects of neonatal herpes, readers are directed to the September 2009 ASCP Forensic Pathology CheckSample.[5] When confronted with rapid decline in a newborn, the TORCH infections should figure prominently in the physician's differential diagnosis. These infections include toxoplasmosis, human immunodeficiency virus (HIV), syphilis, rubella, cytomegalovirus, and HSV. Herpes simplex virus is a common pathogen among them; one analysis showed a rate of 55 cases per 100,000 live births.[6] These cases can present an especially difficult challenge to clinicians because the patients' symptoms are often nonspecific,[7] and the maternal history often fails to elucidate the cause of disease. Although most neonatal infections result from exposure to HSV-2 in the genital tract during delivery, 70% of mothers of infants with HSV infection lack any clinical history of infection.[8]

The proliferation of HSV can result in widespread and devastating effects. After replicating in mucosal membranes or the skin, HSV can cause vesicular lesions of the infected tissue and can proceed to infect the innervating neurons. The virus can remain latent for a period of time and then become reactivated and proliferate. In either initial or repeat proliferation, HSV can cause blindness, encephalitis, and life-threatening disseminated visceral infection in immunosuppressed persons and in neonates, as in the present case.

The gross findings in neonatal HSV infection can vary greatly from patient to patient, and none is pathognomonic for the disease. The current patient's autopsy revealed certain characteristics that can support the diagnosis of HSV infection, although none of them is specific for the disease. The pale tan-yellow discoloration of the myocardium and lungs as well as the pale discoloration of the skeletal musculature suggest an underlying pathologic process, although they may simply be due to anemia. Diffuse ecchymosis noted in the anal-genital region and under the scalp can be attributed to sepsis-induced disseminated intravascular necrosis,[9] and edema in these regions can be attributed to the increased endothelial permeability associated with sepsis.[10]

The histologic features of HSV are classic and provide the most helpful clues in rendering a definitive diagnosis. Perhaps the most familiar aspect of HSV is large, eosinophilic Cowdry type A intranuclear inclusions; these are evident in the medium- and high-power H&E-stained slides from the liver of the current patient (Images 1 and 2). These inclusion bodies, which stain pink to purple with H&E, consist of virions and virion fragments[11] and are a tell-tale indication of HSV. This finding is often accompanied by the marginalization of host cell chromatin along the edges of nuclei,[11] giving the nuclei a "ground glass" appearance. The cells that exhibit these characteristics may fuse to form multinucleated syncytia, the presence of which is another diagnostic feature of HSV.[11] These cells can be stained with antibodies specific to HSV and identified by immunohistochemistry or tested for viral DNA using in situ hybridization (ISH) or polymerase chain reaction (PCR). Alternatively, HSV can be isolated from tissue specimens and grown in culture cells. If the patient's serum is available, antibodies to HSV can be measured in serum to diagnose an infection. The last 2 tests were performed, with positive results, in the current case.

After the diagnosis of HSV was rendered in the present case, a detailed examination of the lungs revealed viral features consistent with HSV infection, although no classic Cowdry type A inclusions were identified (**Image 4**).

In the normal course of forensic autopsy practice, microscopic analysis with an H&E stain is usually sufficient to diagnose HSV in the neonate or any other patient. In the present case, for example, the classic microscopic manifestations of HSV infection in the liver, along with cytologic features consistent with HSV in the lungs, were enough to arrive at a diagnosis; the positive lung viral culture confirmed it. Other, laboratory-based, tests for HSV offer the pathologist a means of confirming the suspicion of HSV in the rare case that H&E staining

and microscopic examination yield ambiguous results. The choice of a specific secondary method for HSV diagnosis is predicated on the efficacy, availability, ease of use, cost, and means of preparation of each method.

Culture

Tube culture isolation is the criterion standard laboratory test for HSV in acute lesions where there is a heavy viral load.[12] It is, however, significantly less sensitive than nucleic acid amplification methods like PCR and therefore is not as effective where virions are sparse, such as in the CSF in herpes encephalitis or in disseminated HSV. Another aspect of culture isolation that might preclude its use in the forensic setting is that the possibility of a viral infection must be anticipated, and a sample collected at autopsy, before histologic examination is performed. Samples from formalin-fixed tissues will not allow for viral growth in culture, although cultures from refrigerated/frozen samples may be attempted.

Many pathology laboratories, including those at most medical centers, perform HSV culture. If not affiliated with such a laboratory, a forensic pathologist can send samples out to various laboratories throughout the country for culture. A 2010 study by Saeed and Pelosi[13] determined the average turnaround time for such a test to be 3.3 days for a positive result and 8.9 days for a negative result[13]; these times will vary depending on location, laboratory proficiency, and so on. Samples from vesicles should be isolated by swabbing the base of a vesicle so that the specimen contains basal epithelial cells and then inoculating a viral transport medium with the swab and/or fluid aspirated from the vesicle.[12,14] Calcium alginate swabs should not be used and the specimen should not be frozen, but it should be kept cool (4°C) during transport and processed within 48 hours.[12] Herpes simplex virus can be cultured from pieces of fresh or frozen tissue placed in transport medium and sent to a laboratory under the conditions described above for swabbed samples.[15] If the virus is to be isolated from a blood sample, a 5-mL portion of blood should be extracted and placed in a lavender-topped EDTA tube for transport with refrigeration.[16] In the case of herpes encephalitis, PCR is the preferred means of detecting HSV,[17] so HSV is rarely cultured from CSF.

Microscopic Immunologic Techniques

Microscopic immunologic techniques to detect HSV can be a valuable alternative to tube culture isolation in laboratories that are too small to afford to maintain cell lines and those in remote locations such that specimen handling and transportation could inactivate the virus.[12] Antigen detection has been shown to be at least as

sensitive as culture isolation.[18] Antigen detection is carried out on fixed, solubilized cell specimens, using direct fluorescent antibodies (DFA) or immunoperoxidase tests.[12] Both of these tests have the distinct advantage of being able to give a valid result even when active virus is absent or uncultivable.

Autopsy material can be sent to a laboratory for DFA analysis in a sterile container. Alternatively, lesion, lung/tissue, or throat swab specimens, collected in the manner described above, can be sent refrigerated, but not frozen, in universal transport media.[19] The forensic pathologist can prepare his or her own slides by rolling a swab, collected as described above in the case of tube culture specimens, firmly over 1 or more discrete areas on a microscope slide. If a spatula is used to scrape tissue, the material should be applied to a slide in 5- to 10-mm diameter areas.[12] The pathologist should prepare multiple slides so that a laboratory can stain with both HSV-1 and HSV-2 antisera. Immunofluorescent histology tests may be performed using paraffin-embedded tissue samples. Many laboratories perform DFA analysis; the laboratory should be consulted to ascertain how to prepare the specimen. Antigen detection by immunofluorescence is relatively inexpensive and efficient.[20]

Although DFA analysis is suitable for autopsy testing for HSV, a much more commonly employed methodology uses simple immunohistochemistry. Immunoperoxidase reactions, like DFA described above, use antibodies to identify certain specific antigens, usually proteins or macromolecules. The antibodies are associated with peroxidases, enzymes that catalyze reactions to generate a colored product that allows visualization. Although fixation and drying regimens vary widely between practitioners, one study found that the best method is acetone fixation of freeze-dried sections.[21] Other more common preparation methods include formaldehyde fixation followed by paraffin embedding,[22] the method used extensively by surgical pathologists in everyday practice. The availability of immunoperoxidase staining techniques for forensic pathologists varies with locality; however, numerous reference laboratories offer such testing on paraffin-embedded autopsy samples.

In Situ Hybridization

In situ hybridization identifies viral nucleic acid sequences by exposing them to a probe consisting of a complementary nucleic acid sequence and a reporter molecule. The probe serves as a bridge between the sought-after nucleic acid sequence and the reporter molecule. The complementary nucleic acid sequence can be composed of single-stranded DNA, double-stranded DNA, RNA, or a synthetic oligonucleotide.[23] Reporter

molecules include radioactive isotopes and nonradioactive labels.[23] Certain reporter molecules allow direct visualization of samples under a microscope. Again, preparation methods vary widely; one method entails formaldehyde fixation (in a buffered solution for no more than 24 hours to avoid denaturation) followed by freezing in liquid nitrogen.[24]

Polymerase Chain Reaction

Rapid PCR assay technology is the preferred means of diagnosing HSV in the clinic and can serve as a similarly valuable aid in diagnosing HSV at autopsy. Multiple studies have proven that PCR is more sensitive than viral isolation in culture[25,26]; this and PCR's rapidity render it decisively preferable in most settings. Current Centers for Disease Control and Prevention (CDC) guidelines reflect these findings in their recommendation of PCR, especially in the detection of HSV in CSF.[27]

Such considerations from the CDC and others regarding the usefulness of PCR in the diagnosis of neonatal HSV might seem inapplicable to pathologists, since the scope of these publications is purely clinical. One might wonder whether other diagnostic technologies yield better results when tissue samples are more readily available, as is the case in an autopsy. To answer this question, one study analyzed neonatal HSV autopsy tissues from 10 newborns using PCR, histology, and immunohistochemistry. In all neonates, and in both neural and nonneural tissues, PCR identified target HSV DNA sequences more effectively than immunohistochemistry identified HSV antigen, or than histology identified viral inclusions.[28] Another paper comparing PCR to in situ hybridization and immunohistochemistry demonstrated the ability of PCR to detect HSV in neonatal autopsy tissues at least as effectively as the other 2 methods.[29] This study also noted the capacity of PCR to distinguish between HSV-1 and HSV-2 serotypes, which the other 2 methods lack. These studies support the CDC's conclusions regarding the usefulness of PCR in diagnosing neonatal HSV and suggest that PCR is just as applicable in the autopsy setting as in the clinical one.

When the pathologist suspects neonatal HSV based on cytology, but culture results come back negative, PCR should be used.[30] Turnaround time for PCR is relatively short, at 1 to 3 days.[31,32] The reagents and instrumentation for PCR are expensive, making it one of the more costly diagnostic tests mentioned here. Suitable specimens include CSF, body fluid, dermal/ocular specimens, genital specimens (cervix, rectum, urethra, vagina, or other genital sites), respiratory specimens, throat swabs, and brain, colon, kidney, liver, and

lung tissue.[32] Paraffin-embedded tissue samples can be used, but they must be prepared with caution. Tissues to be embedded in paraffin can be stored for a maximum of 24 hours in formaldehyde before the nucleic acid sequences are denatured, rendering samples useless for PCR analysis. The formaldehyde solution should be buffered to a pH within the physiological range to prevent denaturation.[33] Although PCR is not routinely done in the typical hospital laboratory, a number of reference laboratories are able to do it. It is advisable to contact the laboratories about specific specimen collection, packaging, and transportation. Contacting the CDC about sample submission is also helpful and is highly encouraged.

Serology

The use of serologic analysis to detect the presence of HSV-specific antibodies in a serum sample requires a very specific set of circumstances in the forensic setting, and is therefore one of the least widely useful of the methods mentioned here. It may be a last resort, as it allows for diagnosis when other testing methods are impossible, too costly, or yield negative results.[12] The first requirement is the availability of serum. Its procurement requires foresight on the part of the forensic pathologist. Soon after the patient's death, the pathologist must request a blood sample left over from the patients' hospitalization, although extraction of serum from postmortem blood is problematic but not impossible. Another prohibitive factor is that the use of serology depends on the availability of HSV antibody-specific reagents. However, where these reagents are unavailable, commercial IgG-based test kits can be used. Some of the testing done with these kits has shown 100% sensitivity and specificities of 98% (HSV-1) and 96% (HSV-2) as compared with Western blot analysis.[34]

If the pathologist does attempt to collect the serum sample post mortem, he or she should collect samples aseptically and allow blood to clot at room temperature before centrifugation. Serum should be aseptically transferred to a tightly sealed sterile container, which should be stored at room temperature if storage time will be less than 8 hours, refrigerated at about 5°C if stored from 8 to 48 hours, and frozen if stored for longer or for shipment.[35] The laboratory or commercial test kit instructions should be consulted to ascertain the necessary specimen volume, as the size of the tray well in the specific test will determine how much serum is required.

The **Table** features a list of the various ancillary diagnostic methods described above, along with the samples necessary for their use.

Summary

The current case demonstrates the challenges associated with postmortem diagnosis of HSV infection. In situations in which the routine H&E-stained tissue sections show nonspecific cytologic changes and the viral load is not strong enough for the lung viral culture to grow out HSV, the antigen detection methods described above can be necessary and powerful tools for confirming a diagnosis of HSV.

Table. *Methods of Postmortem Diagnosis of HSV Infection*

Method	Samples
Culture	Vesicle swab; tissue in appropriate viral culture medium; blood in EDTA tube
Microscopic immunologic tests (direct fluorescent antibody, immunohistochemistry)	Lesion, lung, or throat swab; tissue; paraffin-embedded tissue
In situ hybridization	Lesion, lung, or throat swab; tissue; paraffin-embedded tissue; tissue stored in buffered formaldehyde <24 hours before paraffin embedding; tissue frozen in liquid nitrogen
Polymerase Chain Reaction	Lesion, lung, or throat swab; tissue; paraffin-embedded tissue; tissue stored in buffered formaldehyde <24 hours before paraffin embedding
Serology	Serum (preferably from premortem hospitalization)

Abbreviations: HSV, herpes simplex virus; and EDTA, ethylenediaminetetraacetic acid.

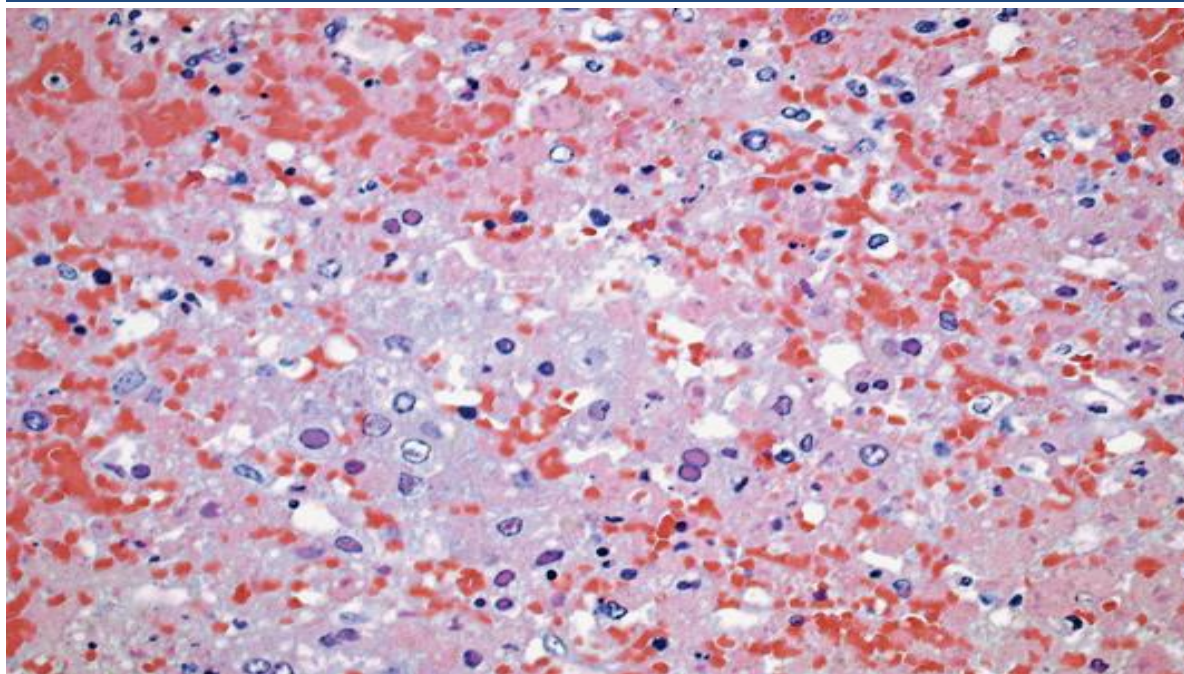

Image 1. Liver tissue hemorrhage and mononuclear infiltration, with cytologic features of HSV infection. Cytologic features include Cowdry type A viral inclusions and the marginalization of host cell chromatin at the outer edges of the nuclei (see **Image 2**) (H&E, original magnification ×400).

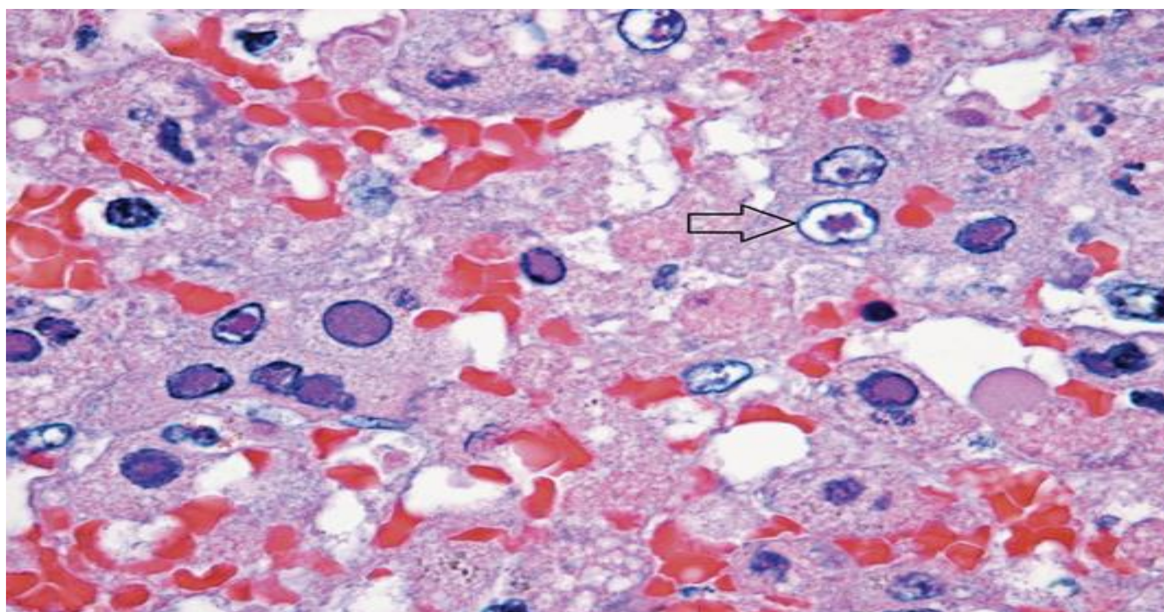

Image 2. Liver tissue with cytologic features of HSV infection, including a cell with Cowdry type A viral inclusions (arrow). These inclusions, composed of virions and virion fragments, are clumped in the middle of the cell. Characteristic halo-shaped clearing surrounding the inclusions and the marginalized chromatin at the outer edge of the nucleus can be seen (H&E, original magnification ×1000).

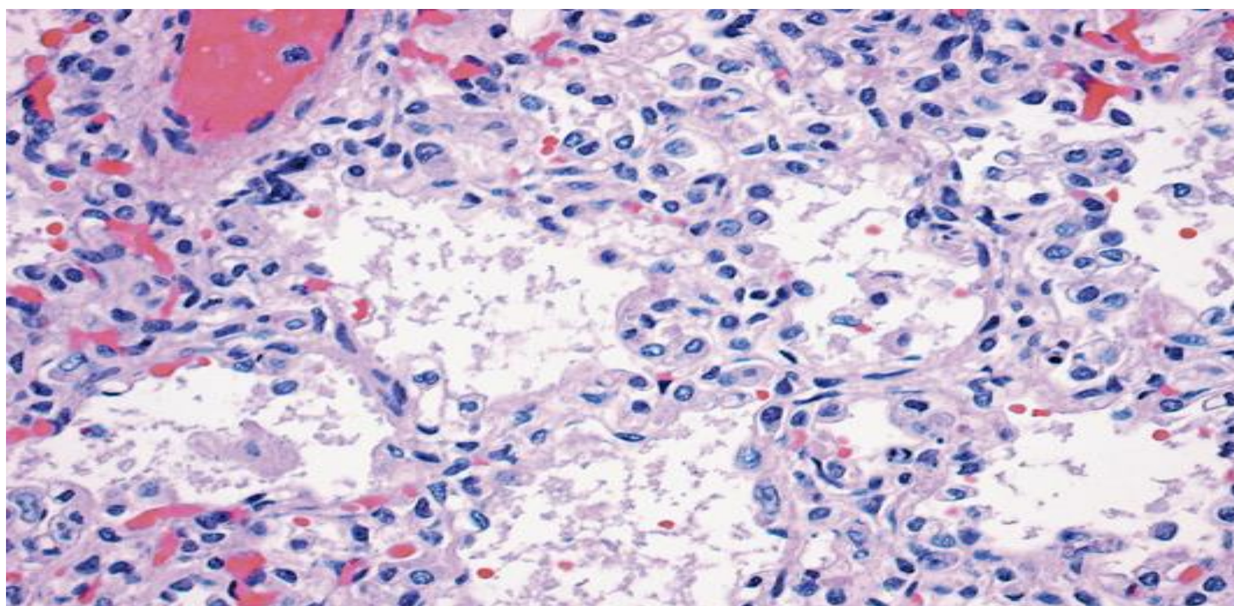

Image 3. Lung tissue with a predominately mononuclear cell interstitial inflammatory infiltrate. Elsewhere within the lung there was evidence of hemorrhage, diffuse alveolar damage, and microthrombi (H&E, original magnification ×400).

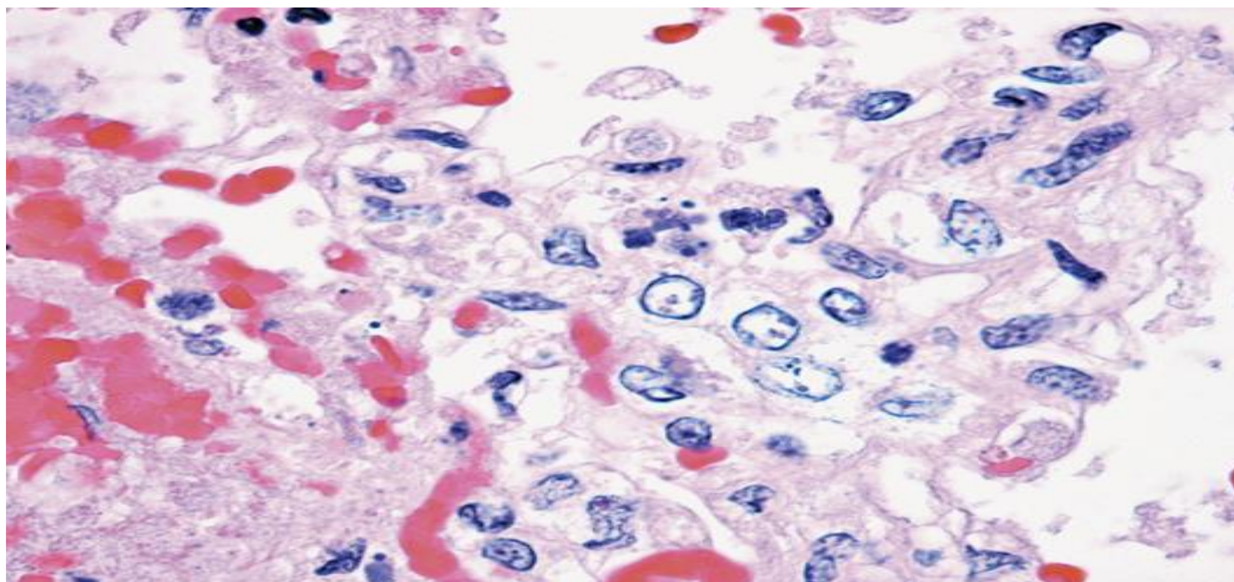

Image 4. Lung section with several HSV-infected cells characterized by nuclei with central areas of clearing containing somewhat subtle eosinophilic inclusions; relatively dense, compressed host cell chromatin can be seen around the nuclear borders (H&E, original magnification ×1000).

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CME QUESTIONS

1. A 3-day-old infant is brought to a clinic with poor intake and a low-grade fever. After a 3-day course of antibiotics, the patient is diagnosed as having pulmonary hypertensive changes and gradually decreasing systemic blood pressure. After clinical deterioration, the infant dies. The pathologist suspects that the patient had one of the most common perinatal infections. Which of the following diseases should be included in the list of possible diagnoses?

   A. Human papilloma virus
   B. Hepatitis A virus
   C. Smallpox
   D. Rubella
   E. Hepatitis C virus

2. A 2-week-old infant dies after a complicated hospital course. A definitive explanation of the infant's severe debilitation and death was never determined clinically. Subtle mononuclear cell inflammation is noted in the lungs and in the liver. Which of the following microscopic findings at autopsy should prompt the pathologist to consider herpes simplex virus (HSV) as a cause of death?

   A. Karyopyknosis and hypereosinophilia
   B. The marginalization of host cell chromatin along the edges of nuclei
   C. Tripolar mitotic figures
   D. Hypercellularity and nuclear hyperchromasia
   E. Acellular pink areas of necrosis surrounded by agranulomatous inflammatory process

3. If a pathologist wants to confirm an autopsy diagnosis of HSV in a newborn, which of the following methods of detection would require that she obtain fresh, nonfixed samples at autopsy, before histologic examination is performed?

   A. Rapid polymerase chain reaction (PCR)
   B. In situ hybridization
   C. Tube culture isolation
   D. Direct fluorescent antibody testing

4. Which of the following antigen detection methods is best if a pathologist is attempting to distinguish between HSV-1 and HSV-2 serotypes to help elucidate the mode of transmission?

   A. Histology with H&E staining
   B. Rapid PCR
   C. In situ hybridization
   D. Tube culture isolation

5. Which of the following is the most appropriate technique to use in the long-term (more than 24 hours) preservation of tissues for antigen detection by PCR?

   A. Paraffin wax embedding
   B. Fixation in formaldehyde solution
   C. Fixation in buffered formaldehyde solution with a pH in the physiological range
   D. Cryopreservation
   E. Fixation in alcohol solution