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Modern Uses of Electron Microscopy for Detection of Viruses

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INTRODUCTION

Electron microscopy (EM) has long been used in the discovery and description of viruses. Organisms smaller than bacteria have been known to exist since the late 19th century (11), but the first EM visualization of a virus came only after the electron microscope was developed. Ernst Ruska, with his mentor Max Knoll, built the first electron microscope in 1931 as the project for his Ph.D. thesis. Eight years later, Ruska and colleagues Kausche and Pfankuch were the first to visualize viruses (tobacco mosaic virus) with the EM (47), and in 1986, Ruska shared the Nobel Prize with Binnig and Rohr, developers of the scanning tunneling electron microscope.

Other historical electron microscopic observations have led to the discovery of new viruses. In 1948, differences between the virus that causes smallpox and the virus that causes chicken pox were demonstrated by EM (62, 92). The first image of poliovirus was taken in 1952 (74), and virus-host relationships began to be studied in the mid-1950s (24, 25). Early virus classification depended heavily on morphology as shown by EM (2, 4, 60), and many of the intestinal viruses were discovered by EM examination of feces after negative staining (32, 46, 54, 96). Cossart et al., in noticing an anomalous reaction while testing normal blood for hepatitis B virus, excised a precipitation band from a gel and, using EM, demonstrated that it contained a very small virus (parvovirus B19) (16). That virus was later determined to be the cause of transient aplastic crisis in patients with sickle cell disease and of "fifth disease," a childhood exanthem. Even today, taxonomy books include electron micrographs of viruses in their descriptions (22).

One of the main advantages of using EM for viral diagnosis is that it does not require organism-specific reagents for recognizing the pathogenic agent. Other tests involving molecular and serological methods require that a specific probe be available for virus identification. In the event of a disease caused by an unknown pathogen, it is hard to know which reagent to pick. On the other hand, EM allows an "open view" (a term coined by Hans Gelderblom) of whatever might be present, while molecular tests require knowledge about the potential agent(s) to determine the correct test(s). EM, though it may not be able to identify a virus beyond the family level, at least points the way for more specific identification by other methods such as biochemical assays for specific pathogens. Another fact to keep in mind is that reagents do not exist for all viruses; when they grow poorly or not at all in in vitro systems, obtaining enough material to produce commercial test kits is difficult. Finally, in cases of dual infections, molecular or antigen-based testing would likely miss the second agent.

Even today, in the age of molecular diagnostics, EM is a mainstay in detecting new and unusual outbreaks. For exam-

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ple, norovirus (Norwalk agent) was discovered by EM (46), and EM continues to serve to confirm infection in quality control of molecular techniques (87). EM was instrumental in elucidating the viral agent of the first outbreak of Ebola virus in Zaire in 1976 (8, 45, 71) and in identifying the Ebola Reston infection of a monkey colony in Reston, VA, in 1989 as being caused by a filovirus (28). In 1999, the causative agent of a strange skin infection in an immunosuppressed patient, coined trichodysplasia spinulosa, was identified by EM as a polyomavirus (36); since then, eight additional cases have been described and confirmed by EM of thin sections of skin biopsy specimens. Further, the Henipavirus (*Hen*dra and *Nipa*h) outbreaks in Australia and Asia were first described by use of EM (15, 42, 44), and in 2003, EM recognized lymphocytic choriomeningitis virus as the cause of fatalities of recipients of organs transplanted from a single donor (23). Much work was done trying to identify the severe acute respiratory syndrome (SARS) agent before it was classified by EM as a coronavirus (20, 50), and the monkeypox outbreaks in the United States in 2003 (6, 31, 75) were discovered by EM to be caused by a poxvirus. Viruses stored in various solutions for extended periods are not viable for culture detection and may be unsuitable for molecular testing. However, EM does not require live or intact virus; it has been used to identify variola virus in infected tissue preserved for decades, in many cases, in unknown solutions (79). Exotic infections in animals have also been identified by EM. For example, a ranavirus was detected in green pythons in the first demonstration of a systemic viral infection in snakes (43), and confirmation of a herpesvirus in kangaroos, initially detected with a consensus herpesvirus PCR, was made by EM examination of the isolate grown in tissue culture (84).

Taking a visual look can sometimes detect an unsuspected pathogen. A novel and previously unknown virus was discovered in fish during routine investigation. The virus replicates in the cytoplasm and has morphological features that resemble viruses in the rhabdo-, corona-, and baculovirus families; it was not further characterized (30). In a different study of the incidence of rotavirus in dairy herds in Brazil, polyacrylamide gel electrophoresis not only identified rotavirus, but in 4 of 63 samples, detected a bisegmented genome. Negative staining of stool specimens from these cattle demonstrated a second population of spherical particles of 37 nm resembling picobirnavirus (10). In a 10-year study of poult enteritis, dual viruses were found; rotavirus-like viruses and small round viruses ranging from 15 to 30 nm were detected, but there was no evidence of coronavirus, which is sometimes seen in human enteric disease (97).

Additionally, because it can be a rapid procedure, EM is on the front line in surveillance of viruses that might be used by terrorists. The rapid-response laboratories in the Laboratory Response Network are paired with EM facilities across the nation. Procedures for identification of potential viral agents of bioterrorism have been published (12, 55), and courses on handling these agents have been conducted at the Centers for Disease Control and Prevention (CDC). A list of EM diagnostic virology laboratories in the world has also been published (37). Thus, the electron microscope is essential in identifying unknown agents of emerging diseases and potential bioterrorism incidents (76). EM is also important in monitoring the effectiveness and the standardization of probe technology for detecting biological threats (72).

Besides its use in diagnostic virology, EM has been and continues to be valuable in elucidating mechanisms of virus attachment and replication. This information can be useful in the discovery and design of antiviral agents and vaccines. Another exciting area in this arena is the ultrastructural examination of virus-like particles (VLPs), which are viral capsids formed by using viral proteins but not genetic material (33, 89, 90). In vitro gene transfer using VLPs has been proposed, and the papillomavirus vaccine in use today is composed of VLPs.

EM METHODS USED FOR VIRUS DETECTION

Current Use of EM in Diagnostic Virology

The samples most frequently received for viral examination in the diagnostic EM laboratory are body fluids, particularly stool and urine specimens, although any liquid sample (e.g., cerebrospinal fluid [CSF], bronchoalveolar lavage fluid, tears, blister fluid, or aspirates) can be processed by negative staining and viewed in a matter of minutes. In the case of fecal specimens, most gastroenteritis viruses do not grow in tissue cultures maintained by routine viral culture laboratories, and molecular or immunological reagents do not exist for all gastroenteric viruses. Thus, EM is the one diagnostic modality most likely to catch all of these agents if present in sufficient numbers. With regard to urine specimens, several reasons still make EM the most beneficial testing modality, even though PCR tests are more sensitive. For example, most adults (~90%) have been exposed to polyomaviruses and would most likely produce a positive PCR test of urine. EM, on the other hand, is not as sensitive, and finding morphological evidence of polyomaviruses in urine appears to signal an increase in virus titer that is significant in the monitoring of polyomavirus reactivation in bone marrow and kidney transplant patients (7, 39, 83, 88).

Negative Staining

Negative staining is a rapid procedure used for viewing small particles, such as viruses, in fluids. For specimen processing, large particles (e.g., bacteria or cell debris) should be centrifuged out at a low speed (e.g., at ~2,000 × *g*) for 3 to 5 min. Either the supernatant is placed directly onto a grid and negatively stained (requiring only 10 to 15 min) or, if enough volume is supplied, the supernatant is ultracentrifuged, and the resulting pellet is resuspended in a few microliters of water and then negatively stained (requiring 1 to 1.5 h) (56, 57). The ultracentrifugation force should be 100,000 × *g*, and the time depends on the amount of sample and the type of instrument used. For example, an Airfuge (Beckman Coulter, Inc., Fullerton, CA) spin of 50 μl in an EM-90 rotor or of 200 to 400 μl in a tube rotor would pellet virus in 30 min. To ensure pelleting of virus in larger volumes (e.g., 2 to 4 ml) in a benchtop ultracentrifuge, 50 min is sufficient. We recommend concentration of all fluid specimens, especially CSF and urine, by ultracentrifugation if enough specimen volume is supplied.

The more fluid specimen provided, the better the chances of finding viruses if they are in dilute concentration. Generally, specimens such as tears and blister fluids are exam-

ined directly without being clarified or concentrated because the volume supplied is miniscule. Other samples, such as CSF from an infant, may be small (e.g., 0.5 ml) but can be concentrated in an Airfuge (e.g., in 200- or 400-μl tubes). Some specimens, such as urine, may be provided in larger amounts (e.g., 2 to 20 ml), and the volume concentrated should be as much as possible, up to 4 to 8 ml, depending on the rotor tube size available.

For viewing particulate specimens such as viruses, a support membrane must be placed onto grids to hold the small particles. Examples of support films include Formvar, Collodion, Butvar, and Pioloform, and they have been discussed in detail (35). Once the grids are coated with the films, they are usually stabilized by evaporating a thin carbon coat over them to render them conductive and keep them from melting in the electron beam. Grids can be coated with the support film and carbon in the laboratory (which ensures freshness) or purchased already coated from an EM supply company. The question with commercially produced grids is their age before shipping to the consumer, since with time, they may lose their ionic charges needed for good specimen spreading.

To ensure a smooth, even spread of sample and stain, the support film must be hydrophilic. A freshly carbon-coated grid will provide good spreading, but older grids may not. Methods for returning hydrophobic grids to a state where they will provide an acceptable stain have been described (35). The best way is to glow discharge them if a vacuum evaporator is available. If one is not available, an easy method is to treat the grids on a drop of 1% aqueous Alcian blue for 5 min and then wash them on 3 to 5 drops of water until the rinse droplets are clear. These procedures can be done just prior to adsorbing the virus suspension. There is some question about how long the grids remain fresh; some publications suggest 30 min. If they are stored in a petri dish sealed with Parafilm, they can remain hydrophilic for several days.

Multiple negative stains have been described in detail (35), with the most popular ones being 0.05 to 2% uranyl acetate, 1 to 2% phosphotungstic acid (PTA), and 0.05 to 5% ammonium molybdate. Different stains are preferred by different laboratories, and the virtues and drawbacks include the following. Uranyl acetate acts as a fixative as well as a stain, and viruses can be viewed intact long after the initial diagnosis. PTA actively degrades some viruses, and while immediate visualization is possible, viruses fall apart in just a few hours after staining. Uranyl acetate is radioactive (alpha radiation, contained by glass) and also precipitates in the presence of phosphate salts; PTA does not. Uranyl acetate and ammonium molybdate give a finely granular appearance and are good for small detail. PTA sometimes outlines fringe/spikes on enveloped viruses better and does not cause positive staining like uranyl acetate can. It is prudent to keep all these stains on hand. If one stain does not work well with a particular sample, another should be tried. Laboratories should perfect a staining method and know its characteristics so that if a bad spread occurs, they will be cognizant of it and reprepare the sample. If investigators are not experienced with the technique, they should consult with someone who is, so that false-negative tests do not result from improper processing.

Thin Sectioning

Thin sectioning is used for cells and tissues because they are too thick for the electron beam to penetrate whole. Any method for embedding tissue for thin sectioning is suitable for virus examination/detection (56, 57). This includes fixation in buffered 2 to 4% glutaraldehyde, washing in buffer, fixation/staining with 1% buffered osmium, washing, and frequently (but not necessarily) subsequent en bloc fixation/staining with 0.5 to 1% buffered or aqueous uranyl acetate. This is followed by dehydration in a graded series of ethanol or acetone solutions and then embedding in an epoxy resin. The main limitation of virus diagnosis by thin sectioning is that if the infection is focal, the sampling might miss the area containing viruses.

Routine thin sectioning usually takes 24 to 36 h (but samples can be processed faster; see below). Cells (e.g., white blood cells, exfoliated cells, or tissue culture cells) can be centrifuged and fixed as a pellet with glutaraldehyde to hold them together. Alternatively, they can be fixed in glutaraldehyde and then encased in 1% molten agar. The agar, if used, should not be fixed in glutaraldehyde because it will become cross-linked so that further processing solutions cannot penetrate it; i.e., agar embedment should follow glutaraldehyde fixation. The pellets are then treated as blocks of tissue for thin sectioning.

Rapid Techniques

Negative staining itself is a rapid procedure, taking only ~15 min for a direct preparation (placing the fluid directly onto the grid). Concentrating the specimen by ultracentrifugation or another procedure is a more lengthy step. Benchtop ultracentrifugation is at 100,000 × *g* for 50 min, and Airfuge centrifugation takes 30 min at 100,000 × *g* (30 lb air pressure). Ultracentrifugation should be used for all fluids, including stool samples if enough volume is sent. The Airfuge may be used to wash and concentrate further pellets obtained with the benchtop ultracentrifuge or to concentrate small amounts of specimens such as CSF from a baby.

Rapid methods whereby sections can be ready for viewing within 2.5 to 3 h have been published. Briefly, tissue blocks are cut very thin (e.g., 0.5 mm), the lengths of time in solutions are decreased, and baking is done at a higher temperature for a shorter time (e.g., 25 min at 95°C) (19). Alternatively, the advent of microwave processing has reduced the time required for staining, dehydration, and embedding (49, 95).

Immunoaggregation

Antibody-virus reactions were first observed in 1941 with tobacco mosaic virus (5), and the term "immune EM" was coined by Almeida and Waterson (4) to describe the clumping of viral particles in EM. Immunoaggregation can be used to increase the sensitivity of virus detection where low concentrations of virus are aggregated so that they may be more easily seen. Aggregation of viruses by antibodies has also been used both to identify viruses specifically and to attract them to grids.

In fluid-phase immune EM, virus is mixed with virus-specific antiserum and centrifuged at a medium speed (e.g., 15,000 × *g*) for 1 to 1.5 h. If the antibody recognizes the virus, an aggregate of viral particles in an immune complex is formed,

thus concentrating and specifically identifying the particles. If the antibody is in excess, many molecules may coat virus particles in addition to or instead of aggregating them. This results in a fuzzy halo around the particles in negative stains. In clinical material, both viral clumps and antibody-coated viruses can be seen if the patient is immunocompetent.

In solid-phase immune EM, antibodies are first attached to the EM grid substrate, either by direct incubation of the grid on dilute antibody drops or by first incubating the grid with protein A, which sticks to the grid. It then attaches to the Fc portion of the antibody, holding the active antibody sites outward. The antibody then attracts and traps the virus particles onto the grid, after which they are negatively stained.

Immune EM has been used to identify elusive viruses that may not be grown in cell culture, such as by using antiserum to detect papillomavirus in wart material (3), and new, previously unidentified viruses have been detected by using convalescent-phase serum to detect norovirus (the Norwalk agent) in stool (46).

Of course, the use of a virus-specific reagent necessitates that one know or at least suspect the type of virus for selection of the proper antibody. However, pooled gamma globulin may be used in an attempt to concentrate viruses when the agent is unknown.

Immunolocalization

Studies with viral antibodies and gold-labeled secondary antibodies have demonstrated the location inside cells of various viral proteins, which sheds light on how the proteins are assimilated. For example, in thin sections, ultrasmall gold probes and silver enhancement established evidence of human immunodeficiency virus (HIV) antisense protein and its association with cellular membranes in infected cell lines (9). In hantavirus-infected cells, immunolabeling showed that the viral inclusions were composed of nucleocapsid protein (29, 99). Additionally, antibodies against engineered proteins can demonstrate differences between them and the native proteins, suggesting the functions of various components. For example, antiserum against a pseudorabies virus protein synthesized in a bacterial system labeled cytoplasmic capsids, as well as intracytoplasmic and extracellular virions, but not perinuclear enveloped virions. This suggests that the protein facilitates tegumentation during morphogenesis in the cytoplasm (48). Finally, for specifically identifying whole viruses, they can be immunolabeled after adsorption onto grids by treating them with primary antibody followed by secondary antibody conjugated with colloidal gold; the grid is then negatively stained.

Biosafety

Handling of all unfixed human specimens should be done under universal precautions (http://www.cdc.gov/ncidod/dhqp/gl_isolation.html) (13, 81) when preparing them for EM. Methods for EM preparation of potential viral samples have been published (55–57). Briefly, all potentially infectious material should be manipulated in a level 2 biosafety cabinet (BSC) using gloves and a lab coat. Unless the laboratory is specialized in handling clinical specimens, it is recommended that grids of negatively stained preparations be disinfected (e.g., fixed) before placing them into the microscope. If the laboratory is accustomed to dealing with infectious organisms, grids can be viewed without this step in cases of necessary rapid turnaround time, keeping contaminated forceps, etc., separate from instruments in general use for other purposes. It should be noted that the electron beam does not kill all of the agents on a grid, nor does drying. If grids are to be kept, they should be inactivated with fixative or with UV light (254-nm wavelength, for 10 min on each side of the grid) before storage.

Bioterrorism Agents

EM is instrumental in the detection of poxviruses in clinical samples and can be used to differentiate variola virus, the causative agent of smallpox, from varicella-zoster virus, a herpesvirus that is the causative agent of chicken pox and shingles. Instructions for specimen preparation are available online (http://www.bt.cdc.gov/agent/smallpox/lab-testing/pdf/em-rash-protocol.pdf), and in the event of a possible bioterrorist release of a suspect agent, specimens can be processed in a level 2 BSC while using biosafety level 3 precautions. All samples are fixed, and all instruments are decontaminated before they are removed from the BSC. Laboratories must be knowledgeable up front about the regulations for becoming involved, including receiving vaccination, before accepting a potentially dangerous agent, and precautions have been described in detail (55).

Cryo-EM and Tomography

Rapid freezing of virus suspensions and examination of the vitrified samples permits examination of non-chemically altered structures (1). Specimens are flash-frozen in liquid nitrogen, transferred to the microscope in a special cold chamber, and viewed frozen in a special transmission electron microscope equipped with a cryo stage. Many digital images are made at different tilt angles and reconstructed by a computer into a three-dimensional representation (52, 73, 85, 86, 93). Digital imaging also permits coloration of different components.

Another procedure that has been described for comparing processes of virus assembly in cells uses an algorithm built from invariant characteristics of viruses seen in conventional thin sections. Alterations of morphology due to mutation or drug treatment can then be detected by comparison with the template (78). Likewise, a semiautomated analysis of digital images based on bispectral features and Gaussian mixture modeling has been described for identifying viruses in negative stains (67).

INTERPRETATION OF EM DATA

Virus Appearance

Numerous atlases (2, 17, 18, 22, 41, 56, 57, 69, 70) and websites with excellent micrographs for virus identification by negative staining and thin sectioning are available. There are also online pictures of viruses (http://www.virology.net/Big_Virology/BVHomePage.html, http://www.virology.net/garryfavweb12.html).

Appearance in negative stains. Human viruses seen in negative stains fall into one of two major morphological categories,

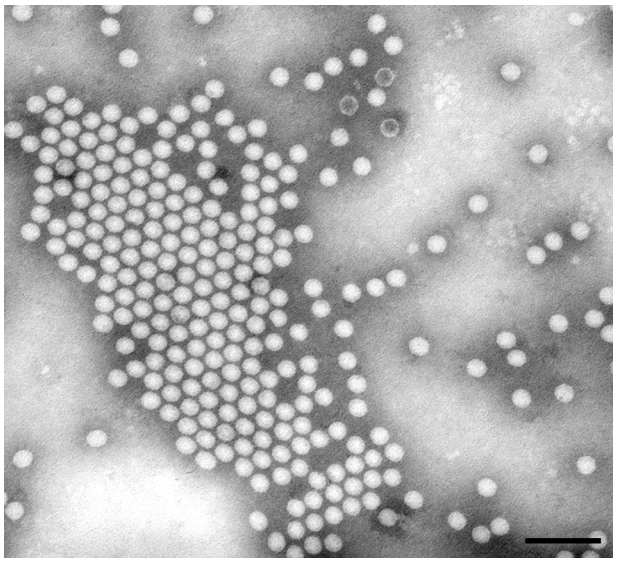

FIG. 1. Negative stain of a small naked icosahedral virus (poliovirus). Bar, 100 nm. Magnification, ×100,000. (Courtesy of Joseph Esposito, CDC.)

naked or enveloped. Naked viruses are icosahedral; their protein coat or capsid is more rigid and withstands the drying process well to maintain their spherical structure in negative stains. Naked human viruses are of three size ranges: 22 to 35 nm (e.g., parvoviruses, enteroviruses, and caliciviruses) (Fig. 1), 40 to 55 nm (polyomaviruses and papillomaviruses) (Fig. 2), and 70 to 90 nm (reoviruses, rotaviruses, and adenoviruses) (Fig. 3). Size is determined in the microscope by comparing the particle to a marker on the screen that represents a known size at a given magnification. In micrographs, size is determined by measuring the particle and knowing the magnification of the picture. A good rule to remember is that at a magnification of ×1,000, 1 mm represents 1 μm; thus, at ×100,000, an object measuring 10 mm is 0.1 μm or 100 nm. At ×100,000, the small icosahedral viruses would be ~3 mm (see Fig. 1).

Enveloped viruses have an outer covering that is usually derived from host membranes. Poxviruses are an exception in that the lipoprotein covering is synthesized de novo, although

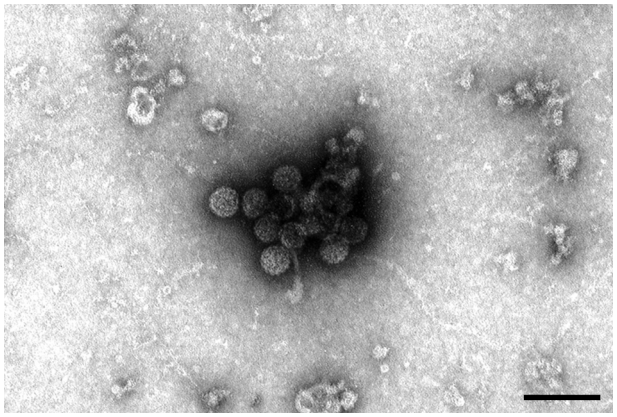

FIG. 2. Negative stain of a medium naked icosahedral virus (polyomavirus). Bar, 100 nm. Magnification, ×100,000.

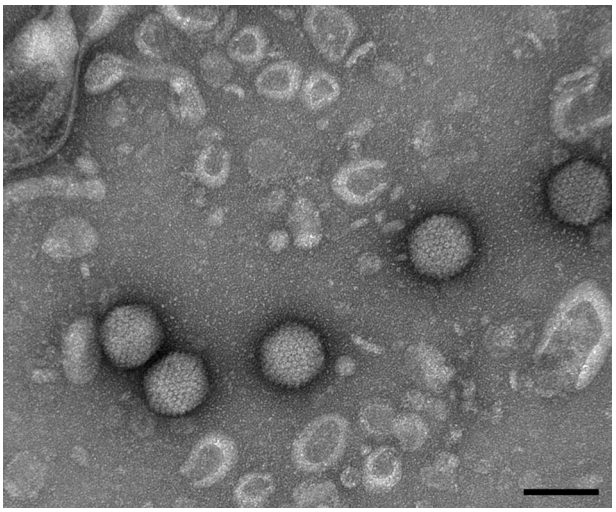

FIG. 3. Negative stain of a large naked icosahedral virus (adenovirus). Note bead-like capsomeric structures that form flat triangular facets on the surface. Bar, 100 nm. Magnification, ×100,000.

sometimes these viruses also acquire an additional host-derived covering. These viruses (except for poxviruses) may take any shape (i.e., are pleomorphic), depending on how they land on the grid and the surface tension of the drying forces. Some enveloped viruses have surface projections long enough to be clearly visualized (e.g., orthomyxoviruses, paramyxoviruses, and coronaviruses) (Fig. 4), while others have short or fragile projections that are rarely seen in negative stains of clinical material (e.g., rubella virus, herpesviruses, and retroviruses) (Fig. 5 and 6).

Enveloped viruses have a nucleocapsid (the nucleic acid held together by some structural proteins) inside. It can be spherical (icosahedral) (Fig. 6), helical (like a Slinky) (Fig. 7), complex (with several components, as in poxviruses) (Fig. 8), or morphologically nondescript (e.g., retroviruses, rubella virus, alphaviruses, and flaviviruses) (Fig. 5).

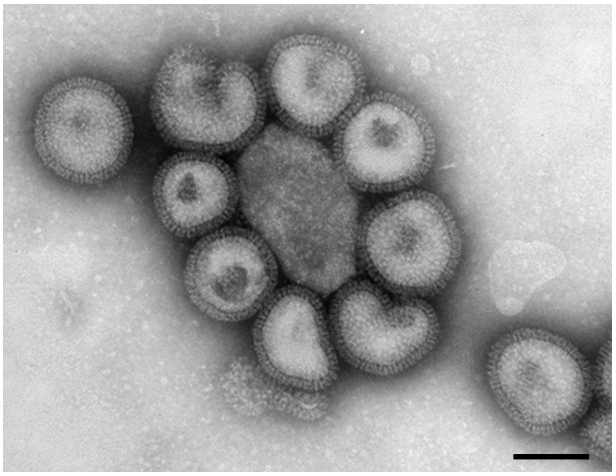

FIG. 4. Negative stain of an enveloped virus with clear surface projections (influenza B virus). Bar, 100 nm. Magnification, ×100,000. (Courtesy of Frederick A. Murphy, CDC.)

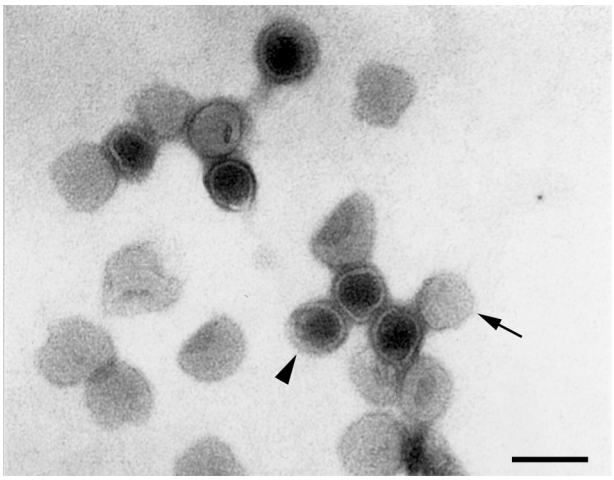

FIG. 5. Negative stain of an enveloped virus with such short surface projections that they are not often visible in negative stains (rubella virus); the nucleocapsids inside are not morphologically distinct. Some particles are outlined by the stain, showing the surface of the virus (arrow), and some are penetrated by the stain (arrowhead) allowing visualization of the interior of the virus. Bar, 100 nm. Magnification, ×100,000. (Reprinted from reference 56 with permission of John Wiley & Sons, Inc. Copyright 1986 Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.)

Appearance in thin sections. Naked viruses cut in thin section may be seen singly or sometimes in paracrystalline arrays (Fig. 9 and 10). They exit the cell by lysing it and are not usually seen attached to and budding from membranes, as are enveloped viruses.

Enveloped viruses are found in association with cellular membranes. They may bud through the nuclear membrane into the cytoplasm (e.g., herpesviruses) (Fig. 11), into intracytoplasmic vesicles (e.g., alpha- and flaviviruses, cytomegalovirus, and coronaviruses) (Fig. 12), or out of the plasma membrane (e.g., herpesviruses, rubella virus, and measles virus) (Fig. 11). The location inside the cell and the type of membranes with which the virus is associated can be clues to identification.

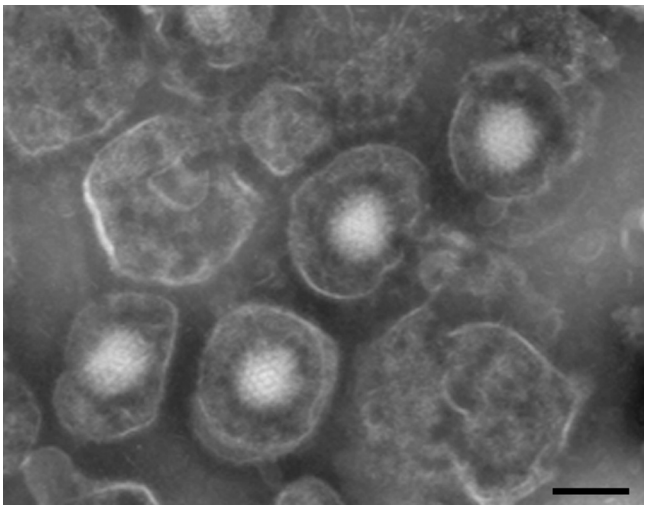

FIG. 6. Negative stain of an enveloped virus with icosahedral nucleocapsid (herpesvirus). The envelope has surface projections that are not readily visualized in clinical material. Bar, 100 nm. Magnification, ×100,000. (Courtesy of Erskine L. Palmer, CDC.)

A general rule of thumb for human viruses is that DNA viruses (except poxviruses) are constructed in the nucleus and RNA viruses are assembled in the cytoplasm. Enveloped DNA viruses can obtain their envelope by budding through the nuclear membrane or by making their way to the cytoplasm and budding into cytoplasmic vesicles or through the plasma membrane. Naked DNA viruses may be seen in the cytoplasm after the cell's nucleus starts breaking down. If icosohedral viruses are seen in the nucleus, they contain DNA; if icosahedral viruses are seen in the cytoplasm, the next step in identification would be to look in the nuclei of cells to see if they are also present in the nucleus.

RNA viruses are not found in the nucleus, with one exception; occasionally, helical paramyxovirus nucleocapsids (but not whole enveloped virions) may be seen in the nucleus. Enveloped RNA viruses can obtain their outer membrane by budding into cytoplasmic vesicles or out of the plasma membrane.

Many animal viruses conform to these patterns. Exceptions include poxvirus and iridovirus, a DNA virus seen in fish, frogs, and some insects, which multiplies in the cytoplasm. Viruses of lower forms do not conform to this generality of DNA virus replication in the nucleus.

Diagnostics

EM laboratories can receive different types of specimens for diagnostics. For example, a public health laboratory may deal mostly with viral cultures and human body fluids. The EM laboratory at Duke University Medical Center processes over 1,000 specimens per year for virus detection. Approximately 80% of them are body fluids, with an occasional tissue culture supernatant for negative staining; the remaining 20% are tissues and cells for thin sectioning. Hospital EM laboratories can monitor polyomavirus excretion in urine of bone marrow and kidney transplant patients by negative-staining EM as an indication of polyomavirus infection (39, 40, 61, 82, 83, 88) and can distinguish it from adenovirus or other virus infections. It is faster than PCR, and at some hospitals, PCR tests have to be sent to a referral laboratory. EM results from fluid specimens are routinely reported to the attending physician in 1 to 2 h, and therapy can be immediately initiated (e.g., in the case of polyomavirus, changing or reducing the immunosuppression). Also, numerous stool specimens from patients with gastroenteritis are received, as enteric viruses do not readily grow in cultures customarily maintained by virology laboratories. In bronchoalveolar lavage specimens, the yield of viruses from the fluid phase is generally low; for this reason, the exfoliated cells are pelleted out and embedded for thin sectioning. Other samples received in a hospital setting include biopsy tissues, cells pelleted from fluids, and occasionally tissue cultures inoculated with clinical material. The turnaround time for thin-sectioned specimens is usually around 24 to 36 h, unless a rapid procedure is used in emergency situations.

In addition to detecting or identifying viruses, EM can sometimes elaborate ultrastructural differences in the morphologies of similar viruses. For example, differences in Marburg and Ebola viruses, both of which are in the filovirus family, have

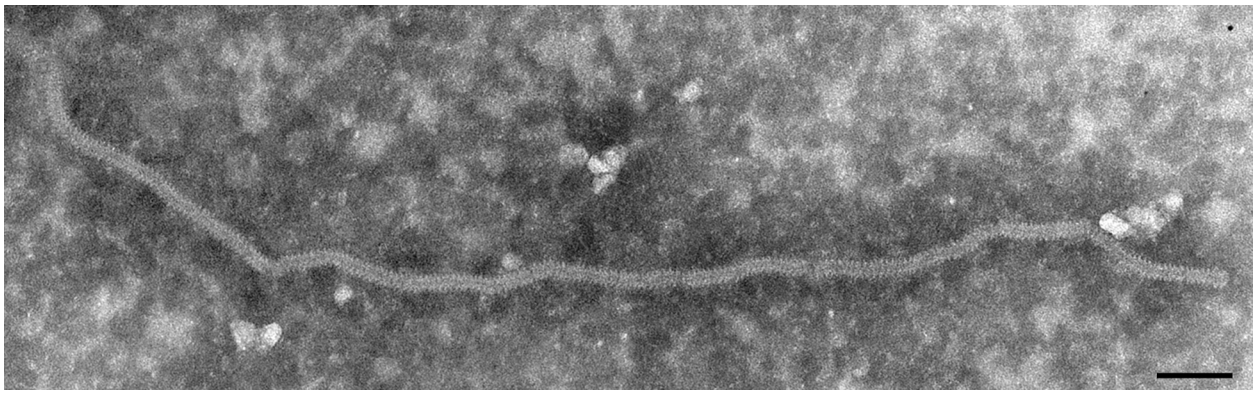

FIG. 7. Negative stain of a helical (like a Slinky) nucleocapsid of Nipah virus. Bar, 100 nm. Magnification, ×100,000.

been demonstrated (27). Marburg virus virions are shorter than those of Ebola virus, and their surface spikes differ. Further, those authors showed some similarities between nascent filovirus inclusions and proviral inclusions of paramyxoviruses.

Pathology

Beyond simply detecting viruses in clinical specimens, the study of virus effects on cells and tissues provides important information on which cells and organ systems are involved and how viruses cause disease. For example, EM of monkeypox virus infection in cynomolgus monkeys determined that death was due to fibrinonecrotic bronchopneumonia and that systemic dissemination was via a monocytic cell-associated viremia. Guarnieri-like bodies were seen in epithelium of the oral mucosa, intestinal mucosa, and skin (100). In a different example, a virus that causes hepatitis and splenomegaly in chickens was shown by EM to be a nonenveloped particle of 30 to 35 nm, similar to hepatitis E virus (34). Genetic studies then indicated that this virus is related to but distinct from human hepatitis E virus. This information is useful in comparison of the disease in animal models to that in humans.

Ultrastructural/Functional Use

Besides clinical diagnostic use, EM is important in the study of ultrastructural features of viruses, which in turn is useful in elucidating the function of various viral components. Proteins on the surface of viruses are responsible for their attachment to and entry into cells (27) as well as for their ability to elicit an immune response (26). Proteins in poxvirus have been altered genetically, and the effect of the alteration on the location inside virus factories in the cells has been studied by thin sectioning (53); detecting the association of these proteins with other proteins contributes to knowledge of antigenicity and hence vaccine production. In a different example, a matrix protein was shown to be important in the transport of Ebola virus nucleocapsid proteins to the cell surface and their incorporation into enveloped virions. The budding mechanism of this virus has been studied by EM in order to shed light on ways to prevent budding with antiviral drugs (65).

Negative staining of subviral particles can elucidate the function of different proteins in constructing the capsid and holding it

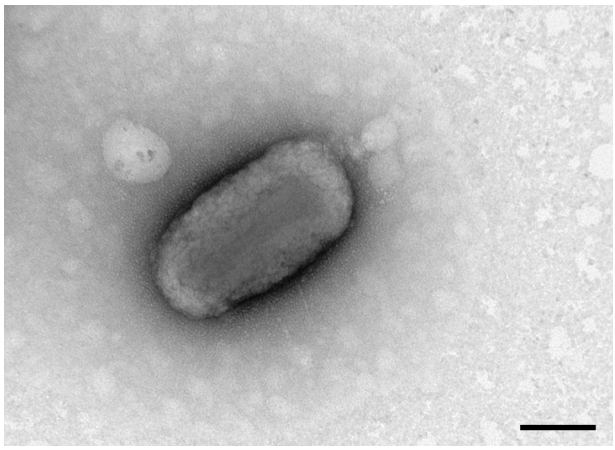

FIG. 8. Negative stain of a poxvirus particle where the surface is covered by short filaments. Bar, 100 nm. Magnification, ×100,000.

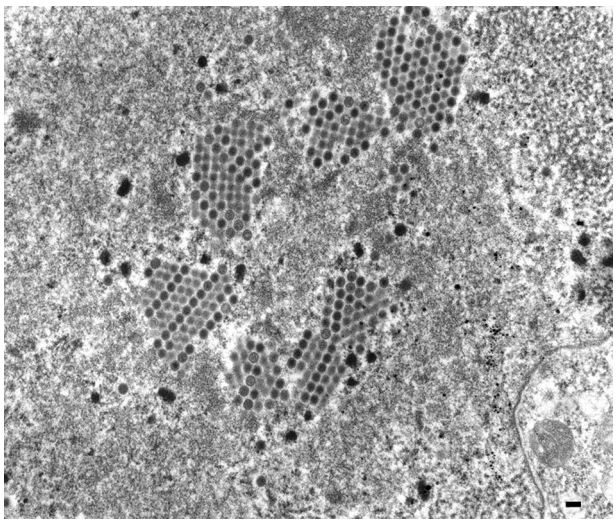

FIG. 9. Thin section of a paracrystalline array of a naked DNA virus (adenovirus) in the nucleus of an infected cell. Bar, 100 nm. Magnification ×20,000.

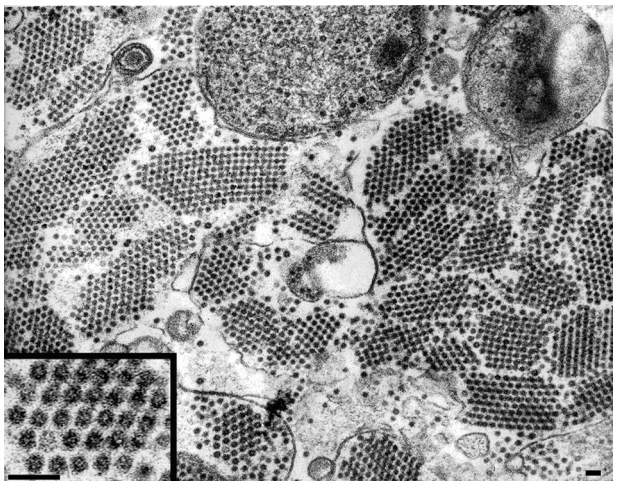

FIG. 10. Thin section of a naked RNA virus (Nodamura virus) produced in the cytoplasm, here seen in paracrystalline arrays. Bars, 100 nm. Magnification, ×20,000. Inset magnification, ×70,000. (Courtesy of Alyne Harrison, CDC.)

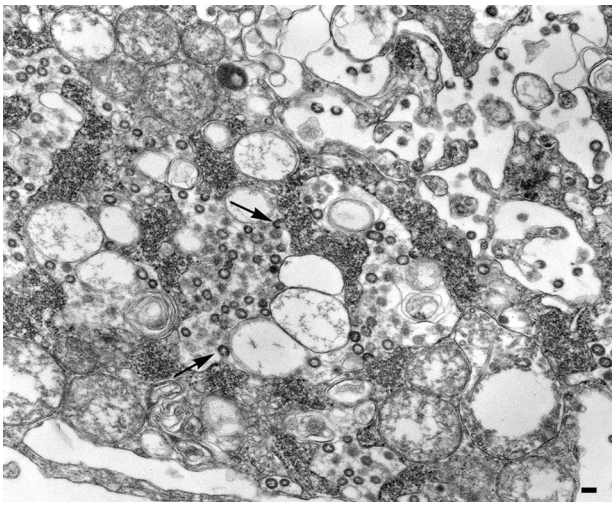

FIG. 12. Thin section of an RNA virus produced in the cytoplasm. SARS coronavirus particles (arrows) obtain their envelope by budding through the membranes of the endoplasmic reticulum. Bar, 100 nm. Magnification, ×20,000.

together. Scaffolding proteins, produced by isolating them on gels or by culturing viruses in the presence of inhibitors, were examined by EM, which showed how the portal complex, the ring-shaped structure at the vertices of herpesviruses, is assembled (63). Earlier studies of reovirus proteins introduced into cells by poxvirus vectors, individually and in various combinations, found particles resembling reovirus cores by negative staining (98).

Cryo-EM and tomography have shown the viral structure of non-chemically fixed virus and provided three-dimensional information (59, 77, 86, 91) (Fig. 13). Even enveloped viruses with considerable pleomorphism, as well as their relationship to subcellular organelles, can be studied. A cell receptor for adenovirus has been demonstrated by cryo-EM, and this has implications in gene therapy (66).

Scanning EM can add valuable information concerning the

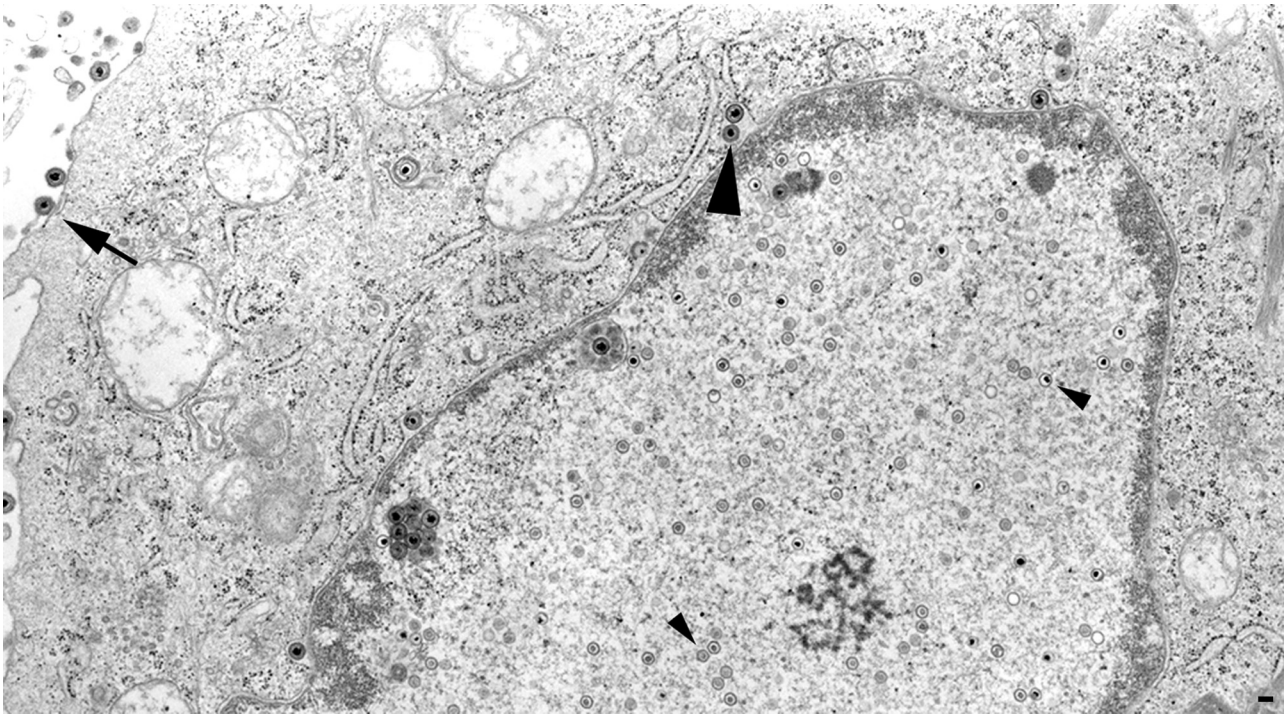

FIG. 11. Thin section of an enveloped DNA virus (herpesvirus). Nucleocapsids are produced in the nucleus (small arrowheads); they can bud out through the nuclear membrane (large arrowhead) to obtain their outer covering, or sometimes they make their way into the cytoplasm naked and then bud into cytoplasmic vesicles or out into extracellular space through the plasma membrane (arrow). Bar, 100 nm. Magnification, ×20,000. (Courtesy of Alyne Harrison, CDC.)

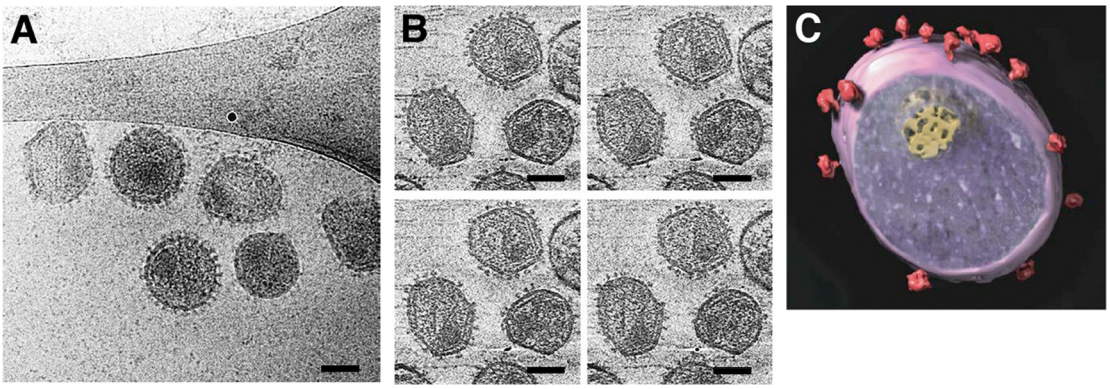

FIG. 13. Electron tomography. (A) Simian immunodeficiency virus viewed frozen hydrated and unstained in a cryo 300-kV transmission electron microscope; glycoprotein spikes and the internal core are visible. (B) Four 1-nm-thick slices from a tomogram. (C) Computer-generated three-dimensional reconstruction of one viral particle seen in panel B. Bars, 50 nm. Magnification, ×100,000. (Reprinted from reference 85.)

exterior of infected cells. For instance, HIV particles accumulate on the surface of HIV-infected cells, and budding virions are sometimes seen (Fig. 14). As another example, the three-dimensional appearance of the SARS coronavirus elucidated the trimeric structure of the 12- to 20-nm surface spikes (51). Knowing how viruses attach to cellular structures and egress from the host sheds light on compounds that can prevent these processes.

The effects of a mutation event or antiviral agent on virus production can be detected and monitored by EM (94). The more we know about virus replication, the more insight we have about methods for preventing replication by using drugs that alter these processes and for designing vaccines that will produce an effective immune response.

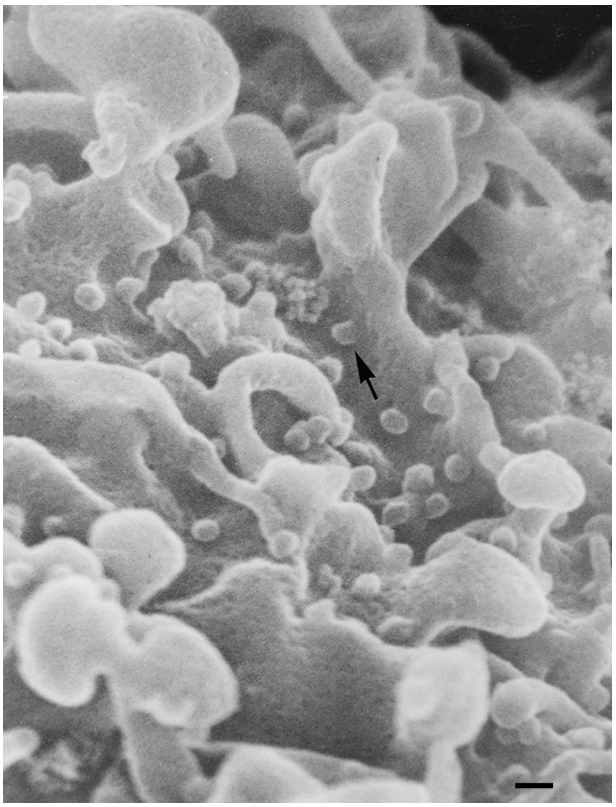

FIG. 14. Scanning EM image of HIV budding from the cell surface of a lymphocyte (arrow). Bar, 100 nm. Magnification, ×50,000.

Use in Detection of Viruses in Tissue Culture

Another important use of EM is the identification of an unknown virus that has been isolated in tissue culture. Viral culture has been one of the gold standards for identification of unknown viruses; this allows for amplification of the virus to a titer that is detectable by EM, i.e., 10^5 to 10^6 particles per milliliter. Even with many molecular tests available, EM is still important in cases where no probes are available. Sometimes endogenous virus or contaminants can confuse the diagnosis of clinical inoculates. These extraneous viruses can prevent the growth of inoculated cultures or confuse diagnosis by producing cytopathogenic effects of their own. For example, cultured monkey kidney cells occasionally are contaminated with simian virus 40, a polyomavirus, and if suspected, it may be detected by immunostaining. If not, simian virus 40, retroviruses, and others can be identified by thin sectioning of the culture and sometimes by negative staining of the culture medium (14, 18, 76, 80). Additionally, mycoplasmas may contaminate cell cultures and result in confusing cytopathogenic effects; they can be seen by EM around the outside of cells cut in thin section (38, 56).

Bioterrorism and Emerging Disease Surveillance

Because of its rapid turnaround time and its ability to detect the unknown and unsuspected organism, EM is on the front line in surveillance of agents in new outbreaks. Guidelines for participation by EM laboratories have been mentioned above.

Caveats

In fluid samples, the concentration of viruses has to be high for detection. Liquids should routinely be ultracentrifuged, after first clarifying them at low speed, to pellet and concentrate viruses. Other concentration methods, including ultrafiltration, agar diffusion, pseudoreplica technique, and immunoaggregation have been reviewed (35, 57).

In tissues, if the pathology is focal, a small biopsy specimen or a single EM block may miss the area of infection. Embedding and cutting semithin sections of multiple EM blocks to examine for tissue pathology is one way to cover more area. It is also possible to examine large (1- to 2-cm) tissue slicer sections of wet tissue by confocal microscopy to select areas of unusual tissue morphology (inflammation, giant cells, necrosis, etc.) for subsequent EM examination (58).

Sample preparation is important in preserving recognizable viruses. Sometimes specimens that are sent for EM examination have not been properly shipped or preserved, and this may cause distortion of viruses and cellular ultrastructure that may be confusing. Drying fluid specimens prior to negative staining distorts viruses. In one report, parapoxvirus was mistaken for orthopoxvirus until another sample prepared from wet material was examined. This has implications in the surveillance of smallpox virus as a potential bioterrorist agent (64). However, frequently, suboptimal specimen preservation can yield diagnostic results. Some viruses (e.g., icosahedral viruses such as adenoviruses and polyomaviruses) can be identified in tissue that has been removed from blocks already prepared in paraffin blocks for light microscopy or even in stained sections on glass slides (21). Thus, the microscopist should be aware of the shipping and storage conditions and consider them in sample examination.

Finally, there are numerous confusing cell organelles and artifacts that may be mistaken for viruses. Examples of cellular components and the viruses with which they can be confused include perichromatin granules (parvovirus), improperly fixed chromatin (paramyxovirus nucleocapsids), nuclear pores and neurosecretory granules (herpesvirus), melanosomes (poxvirus), and cilia (influenza virus). Many other examples have been described in the literature (17, 56, 68).

CONCLUSION

EM is still on the forefront of virus identification, particularly in cases where agents are unknown or unsuspected. It is a valuable technique in the surveillance of emerging diseases and potential bioterrorism agents. Finally, methods for treatment of or vaccination against viral diseases are being investigated through ultrastructural studies that elucidate both viral makeup and the relationship of viruses to the cells they infect.

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