THE YALE JOURNAL OF BIOLOGY AND MEDICINE 62 (1989), 115–130

Electron Microscopy for the Rapid Detection and Identification of Viruses from Clinical Specimens

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Received October 13, 1988

The advantages of using electron microscopy for rapid diagnosis of virus infection from clinical specimens, for identification of virus isolates with unusual properties, and for monitoring endogenous agents in cell cultures are illustrated by several actual cases that have occurred over the years. The importance of using morphological characteristics of viruses for initial identification is emphasized.

INTRODUCTION

With the advent of antiviral chemotherapy, the demand for timely and specific diagnosis of viral infection has become considerably greater than it was a decade ago. Conventional methods for the definitive diagnosis of viral infections consist of culture isolation and serologic confirmation of the etiologic agents. Alternatively, determination of antibody rise in patient sera taken during the acute and convalescent stages of infection may help in the diagnosis of virus infection, although the information may be too late for patient management.

The application of electron microscopy (EM) in the laboratory diagnosis of virus infection offers some advantages which can be utilized favorably for a rapid and accurate diagnosis in various situations. EM can identify both infectious and noninfectious virus particles as well as those viruses that are unable to replicate in conventional cell cultures. EM identification of virus is based on virus morphology and the ultrastructure of virus-cell interaction; it is not restricted by the narrow specificity of viral test reagents used in most rapid immunodiagnostic methods. Furthermore, conventional methods for identification of virus isolates often rely on their biological and antigenic properties. In some instances, a virus isolate does not possess classical characteristics useful for identification but instead has unusual properties, which make identification more difficult when conventional methods are used. In such situations, the use of electron microscopy has provided an alternative approach to rapid and accurate diagnosis.

The following brief review is focused on some practical applications in a clinical

Abbreviations: AIDS: acquired immune deficiency syndrome CPE: cytopathic effect CMV: cytomegalovirus DNA: deoxyribonucleic acid ELISA: enzyme-linked immunosorbent assay EM: electron microscopy HIV: human immunodeficiency virus HPV: human papilloma virus HSV: herpes simplex virus IEM: immune electron microscopy IFA: immunofluorescence assay MCV: molluscum contagiosum virus NS: negative staining PTA: phosphotungstic acid RNA: ribonucleic acid SV-5: simian virus type 5 SV-40: simian virus type 40 VZV: varicella-zoster virus

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laboratory of EM as an approach to rapid, accurate laboratory diagnosis of virus infection. The purpose of this discussion is not to present an extensive review of the literature. Rather, it is intended to concentrate on the unique contribution of EM in its practical application to clinical virology, especially with those viruses possessing unusual features. Therefore, the selection of references is confined to either original discoveries or to review papers.

EM TECHNIQUES FOR SPECIMEN PREPARATION

Two major techniques commonly used in the electron microscopy laboratory for virus study are negative staining and thin sectioning. Detailed descriptions of these procedures can be found in several review papers [1–5].

Negative Staining

Negative staining (NS) has been one of the most useful techniques for visualization of virus particles since its first application in virology by Brenner and Horne in 1959 [6]. It is the method of choice for rapid identification of virus particles in clinical specimens because of its simplicity, rapidity, and high resolution. A suspension of the clinical specimen is deposited on to a Formvar membrane-coated specimen grid and then stained with a 1–2 percent aqueous heavy metal salt solution, such as sodium or potassium salt of phosphotungstic acid (PTA), at pH 6–7. Afterward the specimen is dried and irradiated by ultraviolet light to inactivate the virus. The grid is then ready to be examined. Fluid specimens containing a high titer of virus particles, such as vesicle fluid, stool extract, and serum, are suitable for direct examination using the NS technique. The entire procedure requires approximately 30 minutes. The limitation of this technique is the need for a large number of virus particles ($\geq 10^6$/ml) in the specimen in order to detect the virus. Various methods have been developed to increase virus concentration and hence the sensitivity of the technique.

Methods to Enhance Virus Visualization

Methods routinely used for enhancing virus visualization in clinical specimens are:

Ultracentrifugation: Ultracentrifugation is commonly used to spin down virus particles. A viral specimen should first be subjected to a low-speed centrifugation to remove gross debris. The clarified supernatant is then centrifuged at a higher speed (15,000 *g*) for one hour, as recommended by Almeida [7]. Pellets are resuspended in distilled water, which helps to lyse cellular structures but leaves the virus intact. More recently, a table model Airfuge (air pressure-driven ultracentrifuge) has become available for spinning minute amounts of virus suspension directly on to EM grids [8]; this procedure could increase virus concentration a hundredfold. After centrifugation, the grids can be stained with PTA and examined.

Agar-gel diffusion: This method was first introduced by Kellenberger and Arber in 1957 [9] and applied to concentrated virus for routine EM examination by Anderson and Doane [10]. It is a simple method and easy to use. A drop of viral suspension is placed on a piece of agar block (1–2 percent) on a microscope slide. A Formvar carbon-coated specimen grid is allowed to float upside down on the virus suspension. Water, salts, and proteins of low molecular weights diffuse through the block agar, leaving the virus particles concentrated in the droplet on top of the agar block to adhere to the grid.

Immune electron microscopy (IEM): Immune electron microscopy has been widely used for rapid immunodiagnosis of virus infection. It is also a method for enhancing the

visualization of virus particles for electron microscopic examination [11]. Immune serum containing specific antibodies to the particular virus suspected is mixed with the specimen, followed by one hour of incubation at room temperature in order to allow antigen-antibody reaction to take place. Virus particles form aggregates by reaction with their homologous antibody. Small virus particles such as Norwalk virus [12] and hepatitis A virus [13] from feces often require use of this technique in order to facilitate the detection and differentiation of the virus particles from background materials. The virus-antiserum mixture can be further concentrated by agar-gel diffusion or ultracentrifugation, as described above. Alternatively, the antiserum can be first absorbed to the grid as a solid phase, after which the virus is applied to the grid and then permitted to form antigen-antibody complexes for visualization [14].

For the purpose of immunodiagnosis, following primary antigen-antibody reaction, ferritin or colloidal gold-labeled secondary antibody (antibody against primary antibody) can be applied to the mixture [15,16]. The antigen-antibody complexes are then made visible in the electron microscope. This procedure is useful for identifying viruses of different antigenic types within the same virus group.

Thin Sectioning

Although the method is less rapid, examination of thin sections of virus-infected cells, which have been properly fixed and embedded, provides a more reliable diagnosis, especially when the virus structure is not distinct using routine NS technique. Thin-sectioning technique has the advantage of allowing the observation of virus-cell interaction, which reveals the site of virus replication and maturation in the host cells, information pertinent to the identification of unknown viruses. On the other hand, the disadvantages of the thin-sectioning method are technical: more time for specimen preparation is needed, as are trained personnel with special skills.

The conventional procedures consist of primary fixation with glutaraldehyde or paraformaldehyde (2–4 percent), post-fixation with osmic acid (1–2 percent), *en bloc* staining with uranyl acetate to enhance contrast (an optional step), dehydration with ethanol, infiltration with propylene oxide, embedding in epoxy resin or some other embedding media, thin sectioning, and staining. Detailed procedures can be found in many electron microscopy procedure writings, such as those cited in the references [4,17–19]. In large medical centers today, EM is standard equipment in most pathology laboratories, and personnel with special skills are available to assist in specimen preparation. Therefore, the availability of EM diagnosis for virus infections should not be a major problem.

APPLICATION OF EM IN CLINICAL VIROLOGY

Diagnosis of Poxvirus and Herpesviruses from Vesicle Fluid

The advantage of using EM for the identification of unknown viruses from clinical specimens has long been recognized. Historically, EM has been used successfully to detect virus in vesicular fluid or pustular material for the diagnosis of smallpox and varicella-zoster virus (VZV) [20–21]. EM provides a method for rapid, accurate laboratory diagnosis of virus infection and thus for proper patient management. Although smallpox has been eradicated, other poxviruses, such as vaccinia virus, can cause generalized infection in immunocompromised hosts and require immediate diagnosis. The prevalence of herpes simplex virus (HSV) infection has been increasing in recent years; EM can provide rapid diagnosis of it. Morphologically, poxviruses are

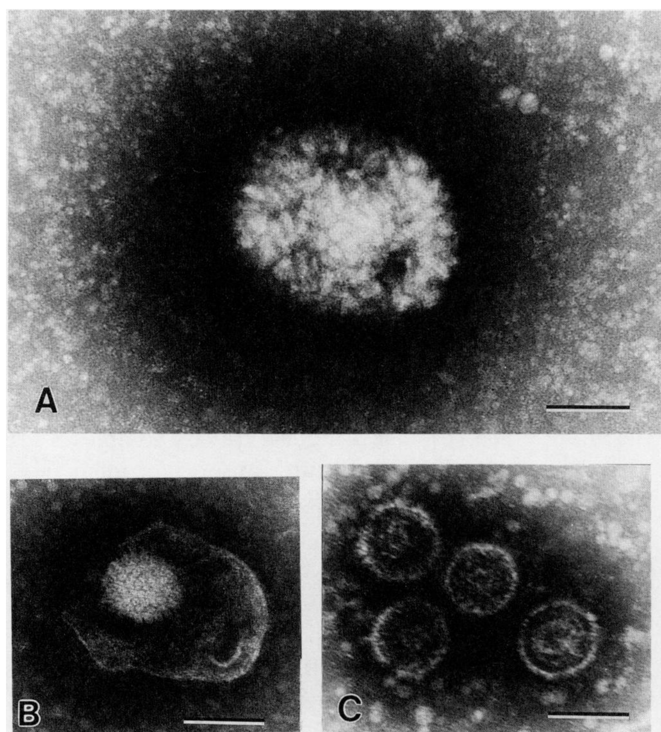

FIG. 1. Viruses in skin lesion, negative-stained preparations. **A.** Vaccinia virus. Bar = 0.1 μm. **B.** Herpesvirus with envelope. Bar = 0.1 μm. **C.** Herpesvirus nucleocapsids without envelope. Bar = 0.1 μm.

large, DNA-containing viruses, exhibiting a brick shape, 300 × 200 × 100 nm in dimension, and with a complex surface structure (Fig. 1A). The herpesviruses commonly causing skin lesions are HSV or VZV. All herpesviruses have the same morphology; they are spherical enveloped viruses with a diameter of 150 nm. Inside each envelope, a unique icosahedral-shaped nucleocapsid, consisting of deoxyribonucleic acid (DNA) and surrounded by 162 capsomers of protein subunits, is easily identified (Figs. 1B, 1C).

Detection of Virus Particles in Stool Specimens from Patients with Nonbacterial Gastroenteritis

In the early 1970s, several groups of investigators simultaneously discovered, by electron microscopic examination, a new virus agent named "rotavirus," which was responsible for many cases of infantile gastroenteritis [22,23]. EM was the only method for rotavirus detection during those years. Morphologically, rotavirus particles are spherical, 70 nm in diameter, consisting of ribonucleic acid (RNA) surrounded by a double layer of capsomers and exhibiting a wheel-like appearance (Fig. 2A).

Subsequently, enzyme-linked immunosorbent assay (ELISA) kits became available for rotavirus detection [24,25]. ELISA only detects rotavirus which shares a common antigen to the virus used in the preparation of the antiserum furnished in the kit; any new viral strain that lacks the common antigen will not be detected by this reagent.

In 1984, we had an unusual experience while examining the stool specimen of a patient from China with epidemic diarrhea: EM examination revealed virus particles with rotavirus morphology, but the specimen was negative by ELISA (Abbott Laboratories). Later in that year, Hung et al. reported a virus, isolated from stool

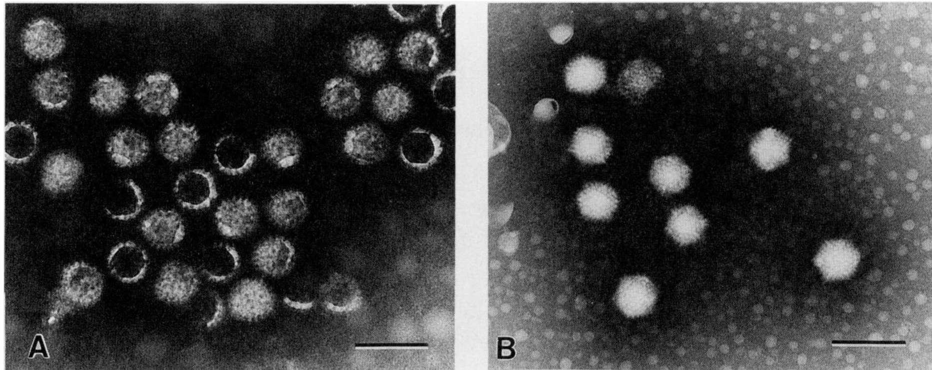

FIG. 2. Virus particles in stool specimens, negative-stained preparations. **A.** Rotavirus particles. Bar = 0.1 μm. **B.** Adenovirus particles. Bar = 0.1 μm.

samples of adult diarrhea patients from China, that resembled rotavirus in morphology, but which, antigenically, lacked the group antigen shared by known rotaviruses [26]. Using ELISA only, without EM examination, our result would have been a false negative. This incident illustrates the advantage of using EM to discover new viruses or viruses known but antigenically different from the prototype strain.

In addition to rotavirus, other virions such as adenoviruses, astrovirus, calicivirus, coronavirus, and enteroviruses are often detected in stool specimens of patients with nonbacterial diarrhea or other gastrointestinal disorders [27–30].

Enteric adenoviruses (types 40 and 41), often associated with infantile gastroenteritis, are 70–80 nm in diameter, with distinct icosahedral symmetry [27–29]. Enteric adenoviruses are often present in stools in large numbers and can be detected by direct electron microscopic examination without difficulty (Fig. 2B). These adenoviruses are difficult to grow in conventional cell cultures. Until very recently, while other immunologic methods of detection were being developed, EM was the only method for detecting these agents in stool specimens.

Norwalk agent, a small, picorna-like virus, 27 nm in diameter, is responsible for outbreaks of gastroenteritis among adults and school children during the winter months [12,30]. Outbreaks have occurred in recreational camps, on cruise ships, in schools, and in nursing homes as a result of drinking or swimming in contaminated water or eating uncooked shellfish [31–33]. Identification of Norwalk virus is done initially by immune electron microscopy (IEM), using convalescent serum containing antibody to Norwalk virus [12].

Virus in Urine Specimens

Cytomegalovirus (CMV) has been frequently isolated from urine specimens of children with congenital infection [34], of immunocompromised patients (including organ transplant recipients), and of patients with acquired immunodeficiency syndrome (AIDS). Isolation of the virus in cell cultures requires from several days to weeks before signs of virus activity become evident. CMV can be directly observed by EM examination, using NS [35,36] or thin sectioning [37]. CMV particles in urine specimens are usually in low concentration; therefore, it is necessary to concentrate the urine specimens by ultracentrifugation. As a supplemental method to tissue-culture isolation, EM can provide a rapid diagnosis of CMV infection. Now CMV early

antigen can be rapidly detected (within 16 hours after incubation of the centrifugation culture) [38]; this technique has an advantage over EM because it is easily adapted in smaller clinical laboratories that lack an EM.

One limitation of the technique is that, in addition to CMV, HSV has occasionally been isolated from the urine of immunocompromised patients. EM examination of NS preparations alone cannot differentiate CMV from HSV because the viruses have the same morphology; observation of thin sections of virus-infected cells for characteristic cytopathology (Fig. 3A) or the use of IEM may aid in identifying CMV or HSV.

Papovavirus is another virus often present but unnoticed in urine specimens of immunosuppressed patients [39,40]. This group of viruses is not usually isolated by conventional methods because of a lack of distinct cytopathic effect (CPE) in cell cultures and a slow replication, requiring two to three weeks to produce CPE. EM can provide a definitive identification, however, because papovavirus has a distinct morphology and can be recognized without difficulty. In papovavirus-infected tissue-culture cells, identification can be achieved by negative staining of the supernatant of infected culture fluid or by thin sectioning of virus-infected cells. Based on the size, shape, and location of virus particles in the cells, one can make a presumptive diagnosis (Fig. 3B).

Our experience indicates that EM is the method of choice for papovavirus identification. In 1981, an unidentified virus isolate was sent to us for EM identification from the Virus Laboratory of Massachusetts General Hospital. This isolate came from a urine specimen of a renal transplant recipient. The virus produced CPE in human embryonic lung and monkey kidney cells, with low virus infectivity titer; the CPE was not characteristic of any of the known common human viruses. The virus was resistant to chloroform treatment, indicating that it was devoid of an envelope. Upon EM examination, this isolate was identified immediately as papovavirus, based on virus morphology.

Direct Examination of Biopsy and Autopsy Tissues

When a virus infection is suspected, a specimen of biopsy or autopsy material can be obtained, fixed, and processed for electron microscopy.

Observation of Virus in Brain Tissues

The availability of chemotherapy for herpesvirus encephalitis prompted the need for a definitive diagnosis of HSV infection, a case of which is illustrated by a thin section of brain tissue in Fig. 4A. Although tissue-culture isolation of HSV remains the most sensitive method for its detection, positive EM identification provides direct proof of virus presence in the specimen and rules out any possibility of laboratory contamination.

Other viruses have been observed in brain tissues, such as papovavirus associated with progressive multifocal leukoencephalopathy [41]. EM remains the method of choice for detection since the isolation of papovavirus in cell culture by conventional methods is difficult.

Human immunodeficiency virus (HIV) can cause neurological disorders in many AIDS patients. HIV particles have been found in their brain tissues [42]. Isolation of the virus in cell culture or the detection of viral antigen in brain tissues may be more sensitive than EM; however, EM examination can produce direct evidence of the viral agent's presence in the target tissue and provide information for an understanding of the mechanism of viral pathogenesis.

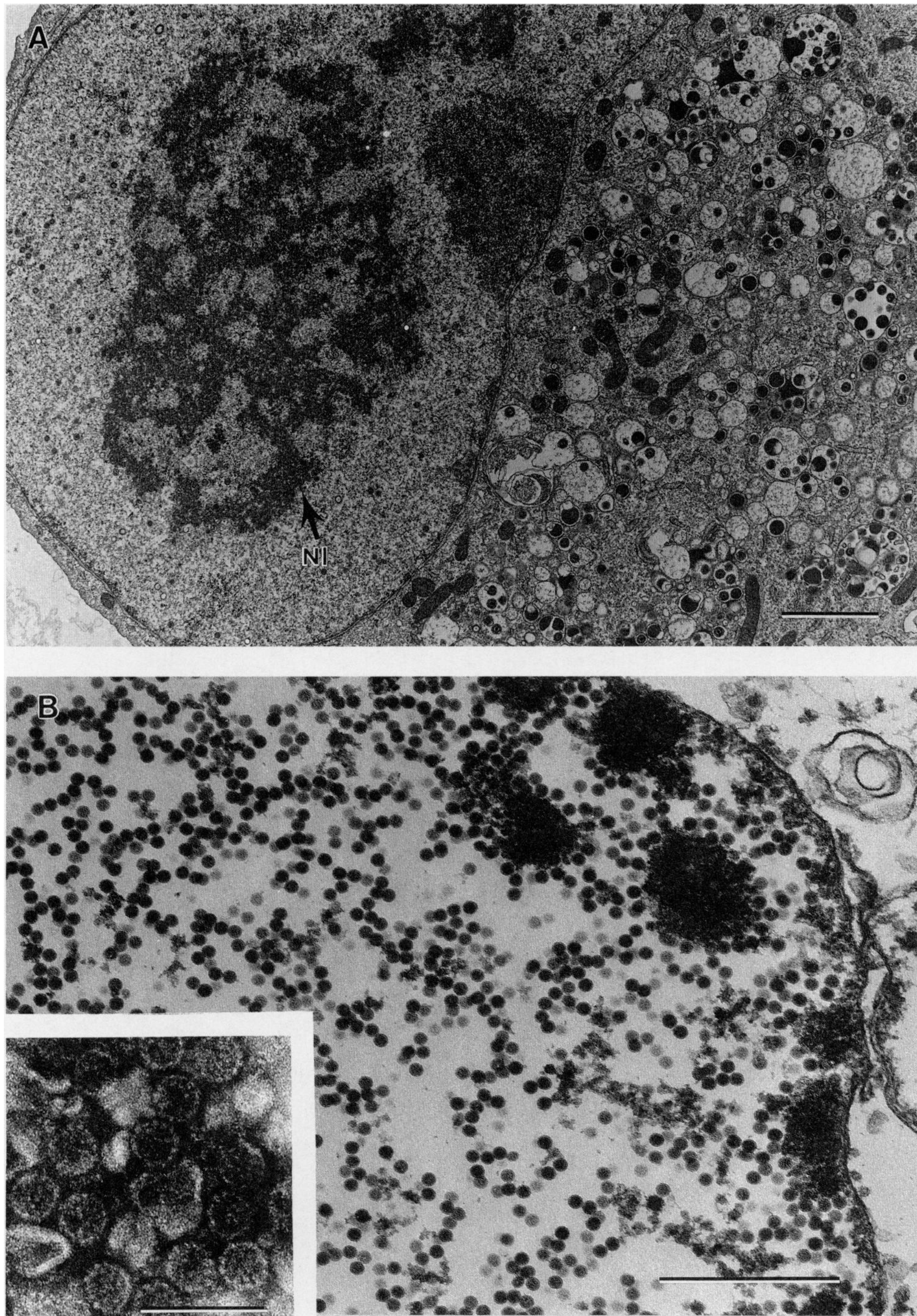

FIG. 3. Virus isolated from urine specimens. **A.** Thin section of cytomegalovirus in infected cells, illustrating a characteristic intranuclear inclusion (N1), and mature virus particles in cytoplasmic vacuoles. Bar = 1.0 μm. **B.** Papovavirus in the nucleus of an infected cell. Bar = 0.5 μm. *Insert* shows the characteristic structure of papovavirus in a negative-stained preparation. Bar = 0.1 μm.

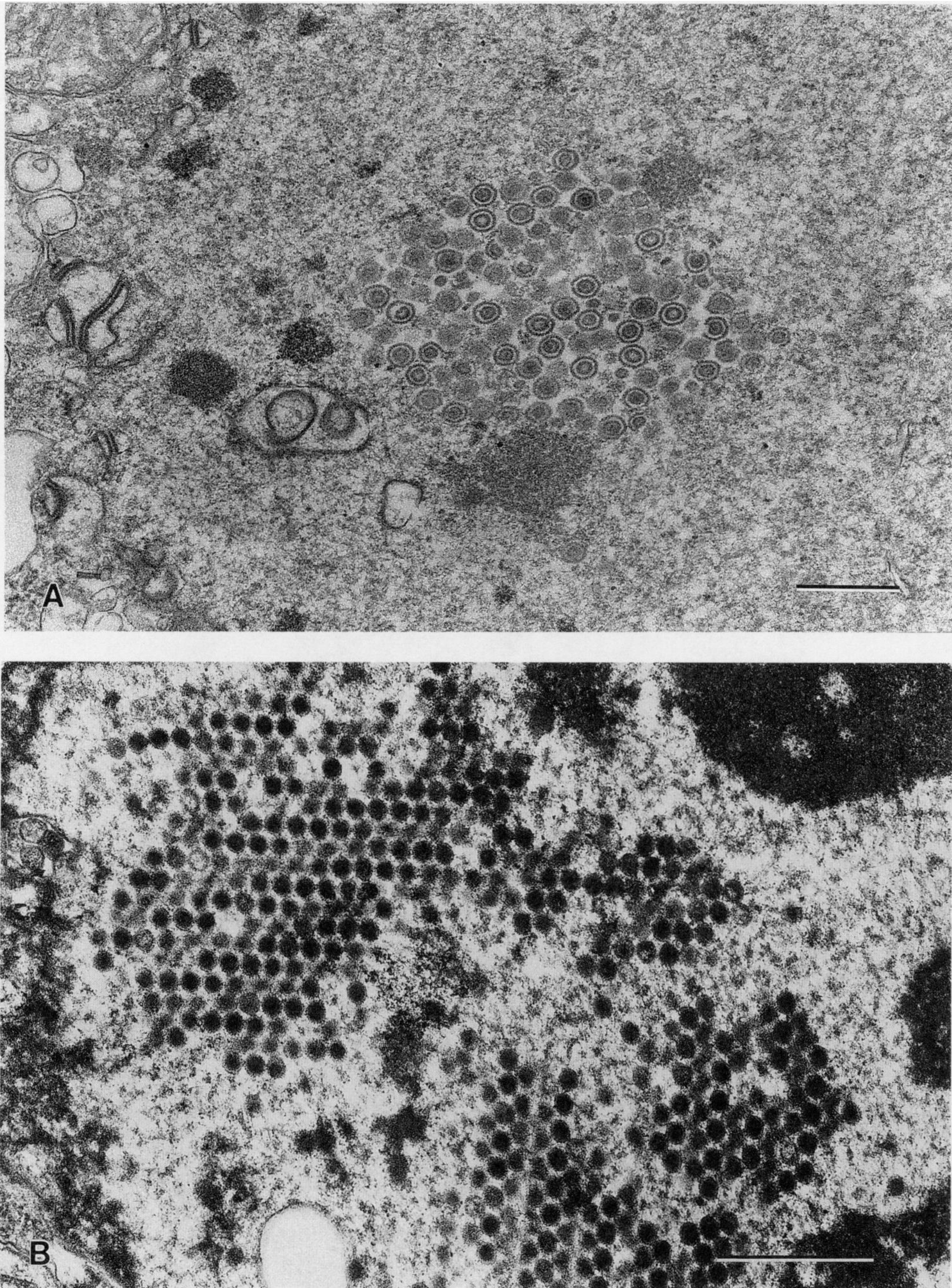

FIG. 4. **A.** Herpesvirus nucleocapsids in the nucleus of an infected brain cell from a biopsy specimen. Bar = 0.5 μm. **B.** Adenovirus particles in crystalline arrays in the nucleus of an infected liver cell from an autopsy specimen. Bar = 0.5 μm.

Observation of Virus from Lung, Liver, and Other Organs

Adenovirus is often isolated from lung and other organs of immunocompromised patients. Adenovirus-infected cells in various tissues show intranuclear inclusions when examined under the light microscope. Adenovirus inclusions can be mistaken for CMV inclusions. To distinguish CMV from adenovirus, EM examination is the preferred method. One of our early experiences in the diagnosis of adenovirus infection occurred when a chronic myelogenous leukemia patient, who had received a bone marrow transplant, developed a rapidly fatal gastroenteritis. Histopathologic examination by light microscopy revealed what looked like CMV intranuclear inclusions in the cells of liver, lungs, and small bowel tissues and was diagnosed as CMV infection by pathologists. Postmortem specimens were also processed for EM and virus isolation in cell cultures. EM examination revealed virus particles that resembled adenovirus but not CMV in the nuclei of infected liver cells (Fig. 4B). Virus isolation showed that this isolate had a broad spectrum of cell susceptibility; it replicated in many types of cell cultures, including primary guinea pig embryo cells, resembling that property of herpes simplex virus (HSV) [43,44]. By tissue-culture neutralization test, however, the final identification of this virus isolate was adenovirus type 2. In this case, EM identification was the key to an accurate diagnosis of this adenovirus infection.

Virus Observed in Skin Tumors

Human papilloma virus (HPV), the etiology of common warts, was first detected through use of the electron microscope by Strauss et al. in 1949 [45] and later confirmed by others [46–48]. HPV belongs to the papovavirus group, with a diameter of 50–55 nm, and exhibits icosahedral symmetry. It propagates in certain epidermal cells in human common warts and appears as intranuclear crystalline arrays [45,47] (Fig. 5A). At present, HPV has never been propagated in any cell culture *in vitro*. For a long time, EM examination has been the only method of detecting the presence of HPV in laboratory diagnosis. Recently, nucleic acid hybridization, using an HPV DNA probe, has become commercially available for laboratory diagnosis of HPV infection.

Molluscum contagiosum virus (MCV) is a poxvirus causing skin tumor. The virus can only be detected by EM examination (Fig. 5B). Similar to HPV, MCV is not capable of replicating in cell culture *in vitro;* EM is the method used for laboratory diagnosis [48].

Identification of Isolates with Unusual Properties

In a routine diagnostic virology laboratory, most of the virus isolates can be identified by their biological properties, such as the appearance of cytopathic changes, hemagglutination activity to avian or mammalian red blood cells, or by antigenic properties, using group- and type-specific antiserum. In some cases, however, identification of isolates possessing unusual properties can be difficult. EM has often provided rapid identification of those viruses with unusual properties.

Identification of Unusual HSV Isolates by EM

HSV is the most common virus isolate encountered in clinical specimens today. Since the availability of monoclonal antibody for typing of HSV-1 and HSV-2 by immunofluorescence assay (IFA), the identification of HSV isolates has become a simpler and easier task than ever before. Once the characteristic CPE of HSV is

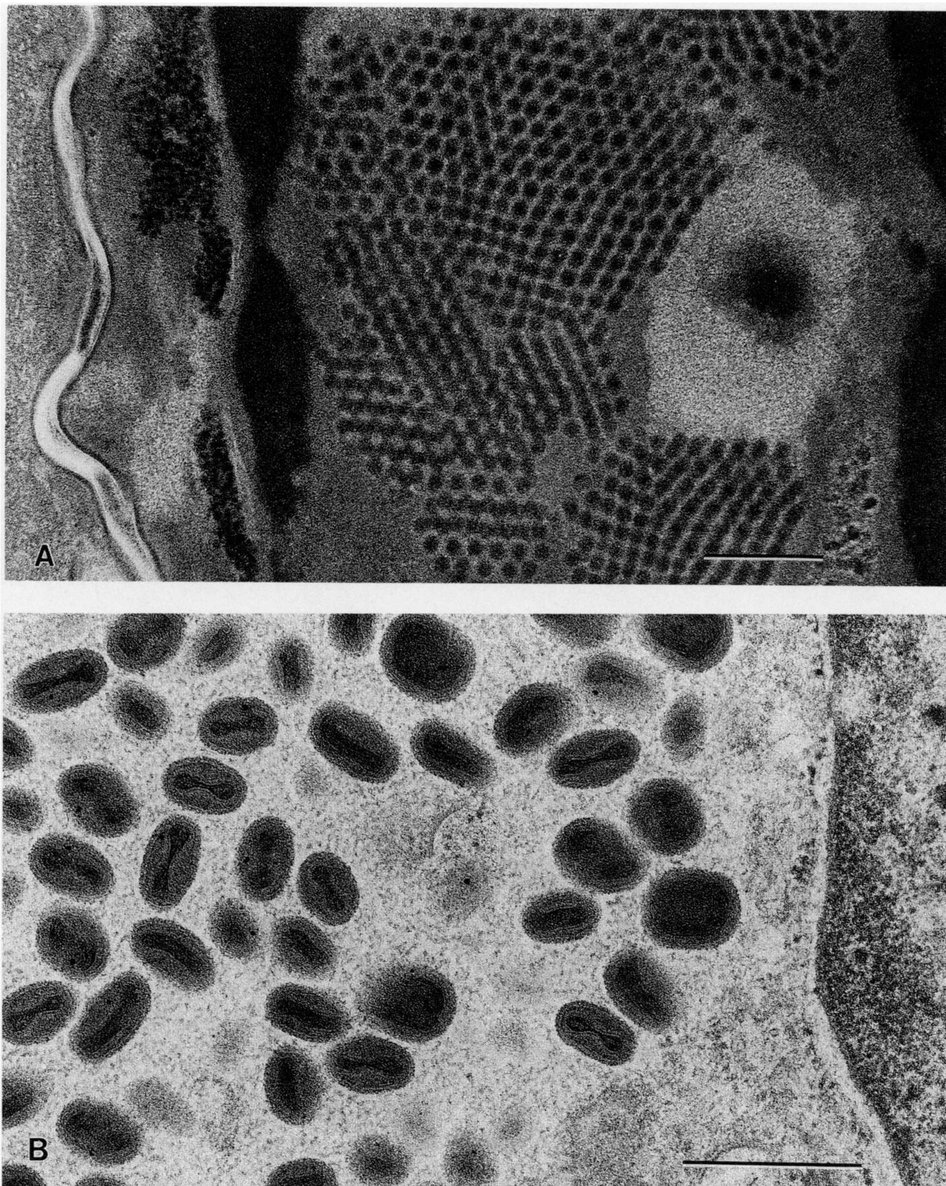

FIG. 5. **A.** Human papillomavirus (wart virus) observed in skin tumor, virus particles in crystalline arrays, in the nucleus of an epidermal cell. Bar = 0.25 μm. **B.** Molluscum contagiosum virus (poxvirus) observed in a skin tumor, virus particles in the cytoplasm of infected cells. Bar = 0.5 μm.

observed, confirmatory typing by IFA can be made immediately. In our experience during the last two and a half years, however, among 682 HSV isolates, there were two HSV isolates which were untypable by use of monoclonal antibody to HSV-1 and HSV-2. These two isolates were finally identified by EM and confirmed by IFA, using polyclonal antibody to HSV. According to EM observation, one of the HSV isolates was type 2, based on the presence of intranuclear fiber structures similar to the characteristics of HSV-2 structure (Fig. 6) previously reported [49,50]. This result

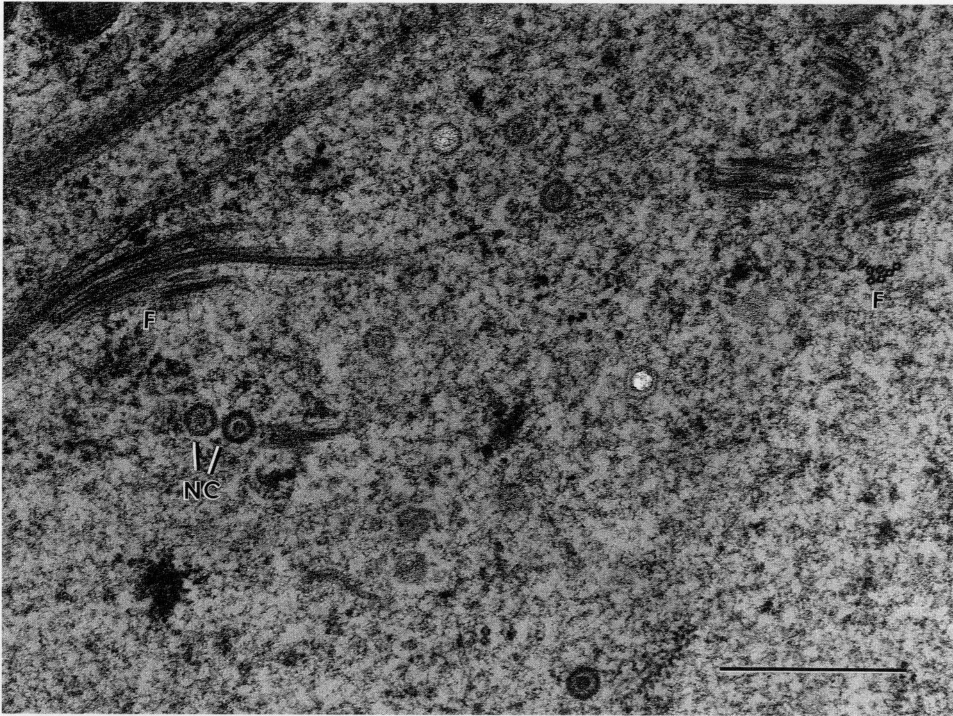

FIG. 6. Herpes simplex virus type 2 identified by EM. Note viral nucleocapsids (NC) and characteristic fiber structures (F) in the nucleus of an infected cell. Bar = 0.2 μm.

was reconfirmed by use of the selective inhibition of (E)-5(2-bromovinyl)-2′-deoxyuridine [51]. In cases of HSV isolates that are not identifiable by monoclonal antibody to either type of HSV by IFA, EM would be very useful as a supplemental method for HSV identification.

Detection of Endogenous Agents in Cell Culture

For virus isolation, clinical specimens are routinely inoculated into susceptible cell-culture systems. Primary cell cultures derived from monkeys or other animals often harbor viruses of their own. Primary monkey kidney cells frequently contain simian virus type 5 (SV-5), simian virus type 40 (SV-40), herpesvirus, cytomegalovirus, and adenovirus of simian origin [52]. It is important that a virologist be aware of the presence of such endogenous viruses in cell cultures being used for clinical specimens.

EM has been used frequently for rapid identification of endogenous agents in cell cultures and calf serum [53,54]. An example is illustrated in Fig. 7: a mixed infection of SV-40 and SV-5 in a monkey kidney cell, which was originally inoculated with SV-5 only, a parainfluenzavirus. SV-40 was apparently an endogenous virus in the cell cultures used for growing SV-5.

McCoy cells, a cell line of mouse origin, are widely used in many laboratories for cultivation of *Chlamydia trachomatis*. This cell line was found to contain C-type virus particles by EM observation (Fig. 8) [Fong CKY: unpublished observation].

Mycoplasma is another common contaminant in cell culture and in calf serum.

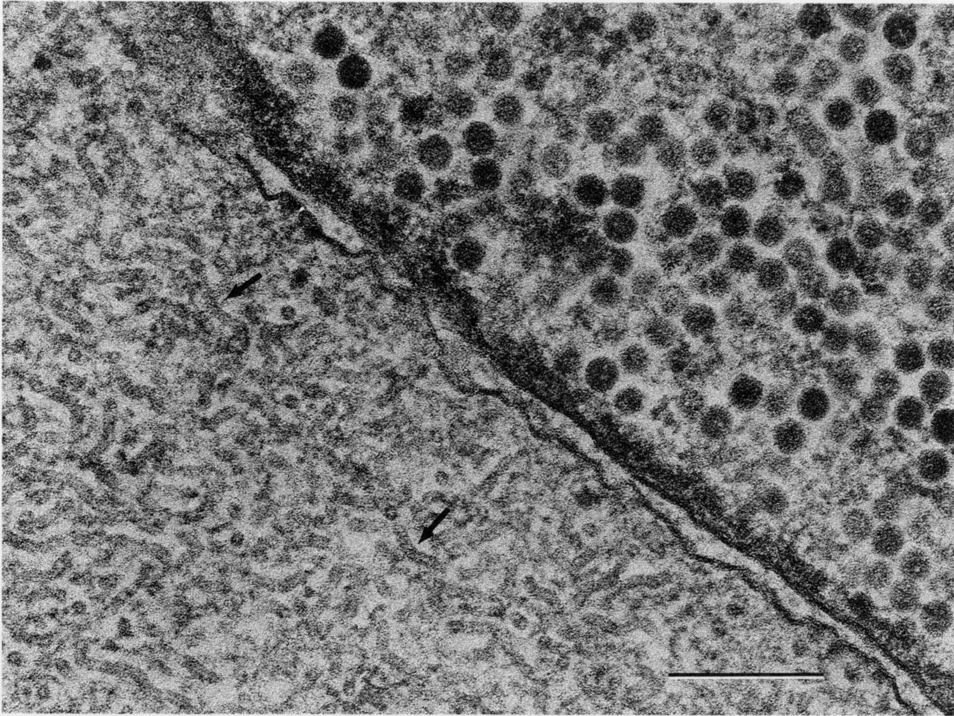

FIG. 7. A primary monkey kidney cell infected with both SV-5 and SV-40. Worm-like nucleocapsids of SV-5 are present in the cytoplasm (*arrows*), and virions of SV-40 can be observed in the nucleus. SV-40 is an endogenous virus from monkey kidney cells. Bar = 0.2 μm.

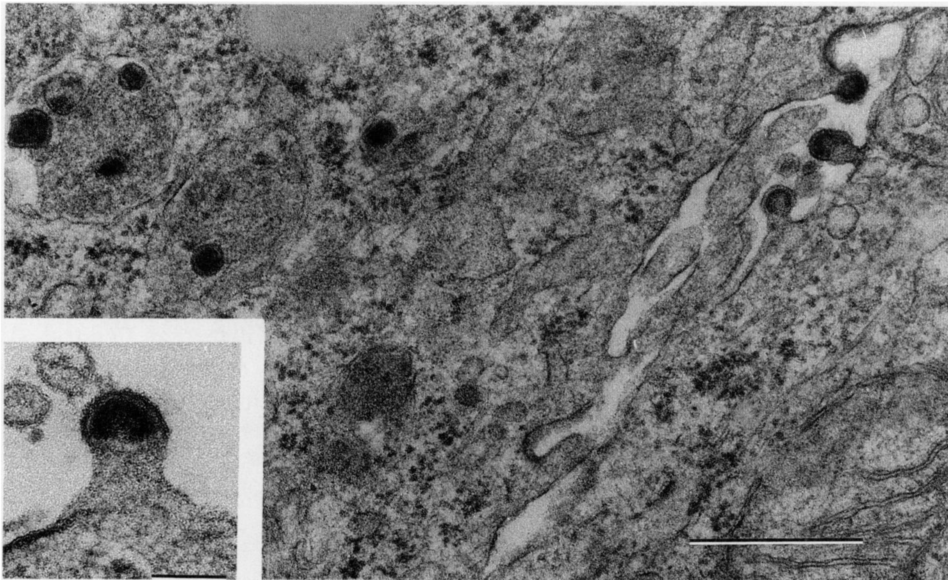

FIG. 8. An endogenous murine C-type virus in a McCoy cell culture; several virus particles are in the process of budding from the cell membrane. Bar = 0.5 μm. *Insert* is a higher magnification of a budding virus particle. Bar = 0.1 μm.

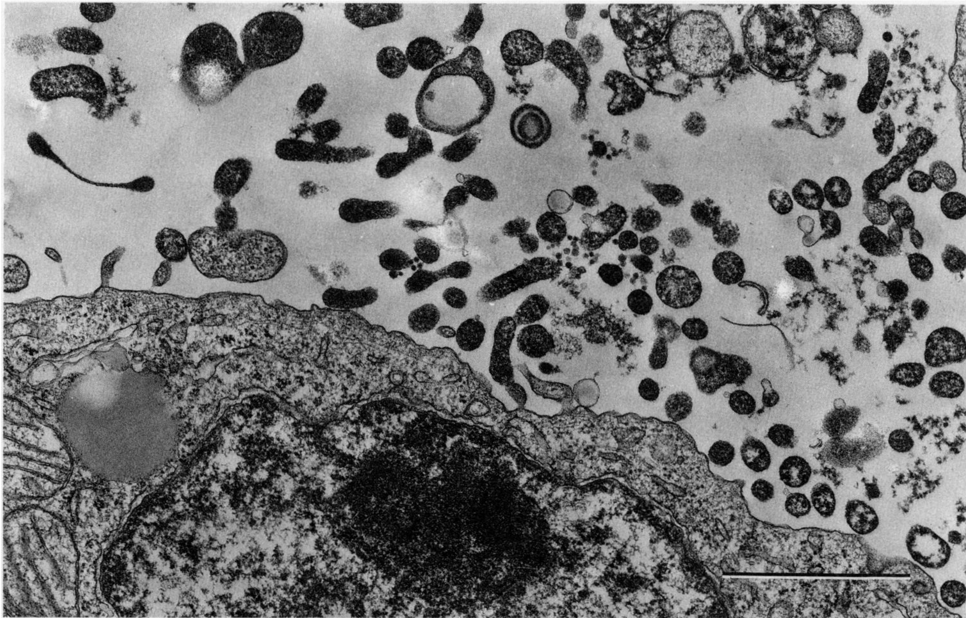

FIG. 9. Cell culture contaminated with mycoplasma. Many mycoplasma organisms with pleomorphic appearance are visible at the extracellular surface. Bar = 1.0 μm.

Although mycoplasma can be detected by cultivation in special medium [55] or by DNA staining followed by fluorescent microscopic examination [56], EM examination of thin sections of contaminated cell cultures often provides a rapid and reliable identification (Fig. 9).

CONCLUDING REMARKS

It is beyond doubt that the application of EM in diagnostic virology has had a great effect on the rapid and accurate laboratory diagnosis of viral infections in certain clinical diseases. In specimens containing a large number of virus particles, such as vesicular fluid and stool extracts, EM examination of negative-stained preparations can provide a rapid diagnosis within an hour. In specimens with a low virus yield such as urine, various methods of concentration can be applied to the specimens and the diagnosis obtained within two hours. For virus isolates with unusual biologic and antigenic properties or unknown etiology, EM can provide a rapid identification of the virus group based on morphological characteristics. For those viruses which cannot be cultivated in cell cultures, EM is the method of choice for their detection. Endogenous viral agents and/or mycoplasma contaminants in cell cultures often produce adverse effects on the routine use of cell cultures for clinical specimens. By employing EM, rapid detection of such contaminants is possible.

In spite of its limitation of low sensitivity, EM has a definite role in the rapid, accurate diagnosis of many interesting clinical diseases of viral etiology. In some instances, EM is the only method of choice.

DEDICATION

This paper is dedicated to Professor G.D. Hsiung on her seventieth birthday. To me, Professor Hsiung is a mentor, an understanding co-worker, and a long-time friend whose continuous inspiration has led me into the field of diagnostic virology, electron microscopy, and research in the diagnosis of human viral diseases.

ACKNOWLEDGEMENTS

The author wishes to give special thanks to Dr. Veronica F. Chan for editing the mauscript, and to Mrs. Sigrid M. Klein for photographic assistance.

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