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Philippe Roingeard

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Roingeard Philippe [*]

*Virus, pseudovirus : morphogen*è*se et antig*é*nicit*é *INSERM : ERI19, CHU Tours, Universit*é *Fran*ç*ois Rabelais - Tours, EA3856, FR*

* Correspondence should be adressed to: Philippe Roingeard <roingeard@med.univ-tours.fr>

Abstract

Viruses are very small and most of them can be seen only by transmission electron microscopy (TEM). TEM has therefore made a major contribution to virology, including the discovery of many viruses, the diagnosis of various viral infections, and fundamental investigations of virus/host cell interaction. However, TEM has gradually been replaced by more sensitive methods, such as the polymerase chain reaction. In research, new imaging techniques for fluorescence light microscopy have supplanted TEM, making it possible to study live cells and dynamic interactions between viruses and the cellular machinery. Nevertheless, TEM remains essential for certain aspects of virology. It is very useful for the initial identification of unknown viral agents in particular outbreaks and is recommended by regulatory agencies for investigations of the viral safety of biological products and/or the cells used to produce them. In research, only TEM has a resolution sufficiently high for discrimination between aggregated viral proteins and structured viral particles. Recent examples of different viral assembly models illustrate the value of TEM for improving our understanding of virus/cell interactions.

Introduction

Most viruses are small enough to be at the limit of resolution of even the best light microscopes, and can be visualised in liquid samples or infected cells only by electron microscopy (EM). However, there has been passionate debate about whether it is useful or useless for medical virology (Curry et al., 1999; Curry et al., 2000a; Curry et al., 2000b; McCaughey et al., 2000a; McCaughey et al., 2000b; Madeley 2000). In this review, I analyse and discuss the benefits of viral detection by EM in various aspects of current and past virology.

The discovery of many viruses and a role in routine diagnosis: the "glory days" of the past

EM was first developed in the 1930s, by physicists in various countries, including Germany in particular (reviewed recently by Haguenau et al., 2003). The first microscope for transmission electron microscopy (TEM), which was also known as a "supermicroscope", was initially described by Max Knoll and Ernst Ruska in 1932 (Knoll and Ruska 1932; Ruska 1987). This microscope had a much higher resolution than the light microscopes of the time, and promised to revolutionise many aspects of cell biology and virology. Helmut Ruska, a medical doctor and brother of the physicist Ernst Ruska (Ruska et al., 1939) rapidly recognised the potential of "ultramicroscopy" for investigating the nature of viruses. Despite the lack of appropriate methods of sample preparation for TEM at the time, several viruses were characterised morphologically and an attempt was made to develop a viral classification based on fundamental science (Ruska 1943). The first use of TEM in clinical virology concerned the differential diagnosis of smallpox (caused by the variola virus of the poxvirus family) and chicken pox (caused by the varicella-zoster virus of the herpes family), using fluid from the vesicles on the patients' skin (Nagler and Rake, 1948). Commercially available electron microscopes became widely available from several manufacturers during the 1960s and 1970s. Medical publications from this time feature large numbers of ultrastuctural investigations in thin sections of many embedded cells and organs (infected or uninfected; Figure 1A). The introduction of negative staining, making it possible to detect viruses from liquid samples deposited on carbon-coated grids and stained with heavy metals salts (such as phosphotungstic acid or uranyl acetate), led to the widespread use of TEM in basic virology and rapid viral diagnosis (Brenner and Horne, 1959; Figure 2, A and B). Negative staining not only makes the virus stand out from the background, it also provides morphological information about symmetry and capsomer arrangement, for example, making it possible the specific identification of viruses, or their classification into morphologically similar groups. Thus, the use of TEM for the study of viruses peaked during the 1970s and 1980s, when it contributed to the discovery of many clinically important viruses, such as adeno-, entero-, paramyxo- and reoviruses, which were isolated from diagnostic cell cultures. Differences in virus size and fine structure were used as criteria for classification (Tyrrell and Almeida 1967). However, TEM failed to detect agents for other diseases, such as hepatitis and gastroenteritis, because susceptible cell cultures were not available for virus isolation or because the virus could not be cultured. However, a major breakthrough was made for these viruses in the 1970s, when TEM was applied to "dirty" clinical samples, such as plasma, urine and faeces (Madeley 1979). The aetiological agents of hepatitis B (Dane et al., 1970) and A (Feinstone et al., 1973) were detected in plasma and stool samples, respectively. Parvovirus B19 was

discovered during a search for hepatitis B virus in a serum sample from a patient (Cossart et al., 1975). The BK virus, a polyomavirus, was first identified in the urine of patients undergoing organ transplantation (Gardner et al., 1971) (Figure 1B). Rotaviruses were also identified as the main cause of epidemic gastroenteritis in humans and animals by this technique (Bishop et al., 1973; Flewett et al., 1973). However, other viruses were found to be responsible for many outbreaks of gastroenteritis. The first of these viruses was the Norwalk virus, identified during a community outbreak of gastroenteritis in Norwalk, Ohio, USA (Kapikian et al., 1972; Kapikian 2000). Viruses with a similar morphology were subsequently discovered elsewhere and called "Norwalk-like" or "small round structured viruses" to reflect the similarity of their appearance on TEM (Caul and Appleton 1982), before being officially renamed "noroviruses" (Mayo 2002). Other viruses from the adenovirus (Morris 1975), astrovirus (Appleton and Higgins 1975; Madeley and Cosgrove 1975) and calicivirus (Madely 76 and Cosgrove 1976) families were also identified in the stool samples of children suffering from gastroenteritis. This large diversity of viruses potentially involved in human gastroenteritis contributed to the use of TEM on negatively stained samples for routine diagnosis by this rapid, "catch-all" method in clinical virology (Figure 2, C and D).

However, by the 1990s, the increasing development of other techniques, such as enzyme-linked immunosorbent assays (ELISAs) and polymerase chain reaction (PCR), had contributed to a gradual decline in the use of TEM for viral diagnosis in cases of gastroenteritis (McGaughey et al., 2000; Biel and Madeley 2001). Indeed, these antigenic and molecular techniques are much more sensitive than TEM, which has a detection limit of between 10^5 and 10^6 particles/ml. These new techniques are also more appropriate for the screening of large numbers of samples, and can now be used to detect most of the virus families involved in human gastroenteritis (Medici et al., 2005; Logan et al., 2006; Oka et al., 2006; Logan et al., 2007). A similar change has also been observed in veterinary medicine, in which ELISAs and PCR have progressively replaced TEM for routine viral diagnosis (Tang Y et al., 2005; Rodak et al., 2005; van der Poel et al., 2003; Guo et al., 2001). In human medicine, EM viral diagnosis for the differentiation of smallpox virus from other viruses present in the vesicle fluids of skin lesions is no longer required, due to an intensive worldwide vaccination programme leading to the successful eradication of the variola virus in 1980 (Henderson 2002). It has been argued that TEM remains potentially useful for viral diagnosis because the variola virus might be used for bioterrorism (Miller 2003; Curry et al., 2006). However, the risk of smallpox reappearing is very small, and even in the unlikely event of smallpox re-emerging, molecular techniques would certainly surpass TEM for its diagnosis.

Identifying emerging or "re-emerging" agents and the control of viral biosafety

The benefits of TEM for resolving diagnostic problems in clinical virology have nonetheless been clearly illustrated on several occasions in the last fifteen years. TEM proved essential for the identification of a new morbillivirus (Hendra virus, belonging to the Paramyxoviridae) in horses and humans suffering from fatal respiratory infections in 1995 in Australia (Murray et al., 1995). A related virus, the Nipah virus, mostly affecting pig farmers in Malaysia, was discovered more recently (Chua et al., 1999). The aetiology of the severe acute respiratory syndrome (SARS) pandemic in Hong Kong and Southern China in 2003 was first identified as a coronavirus by TEM, leading to subsequent laboratory and epidemiological investigations (Drosten et al., 2003, Ksiazek et al., 2003; Goldsmith et al., 2004). A human monkeypox outbreak in the USA in 2003 was also diagnosed only once TEM had been used (Reed et al., 2004). TEM is occasionally useful for the identification of new subtypes of viruses involved in human gastroenteritis, such as adenovirus (Jones et al., 2007a) or picornavirus (Jones et al., 2007b). The role of TEM in clinical virology in recent years has thus changed from that of a routine technique to a support for the identification of unknown infectious agents in particular outbreaks. In such investigations, the underlying "catch-all" principle of this technique is essential for the recognition of an unknown agent. There are also many recent similar examples of the usefulness of TEM for identifying the virus involved in particular outbreaks in veterinary medicine (Prukner-Radovcic et al., 2006; Coyne et al., 2006; Literak et al., 2006; Matz-Rensing et al., 2006; Chan et al., 2007; Maeda et al., 2007; Gruber et al., 2007).

TEM currently plays an important role in controls of the biosafety of biological products. Rodent cell lines are widely used as substrates for producing biological therapeutic molecules, such as monoclonal antibodies, recombinant proteins, vaccines and viral vectors for gene therapy. These cell lines have long been known to contain retroviral elements, because the rodent genome contains many copies of endogenous retrovirus-like sequences (Weiss 1982). Most of the particles produced in cell culture, such as the intracisternal A-type and R-type particles, are defective and are non-infectious. However, other particles, such as C-type particles, bud at the cell surface and may infect non-rodent cells (Lueders 1991). Some murine retroviruses have been shown to be tumorigenic in primates (Donahue et al., 1992). Murine retroviral vectors have been shown to cause leukaemia in children with severe combined immunodeficiency treated by gene therapy with these vectors (Hacein-Bey-Abina et al., 2003). Regulatory agencies therefore recommend the use of a wide safety margin for biological products derived from rodent cells. They also recommend the use of several, complementary techniques — reverse transcriptase assays (reverse transcriptase being specific to retroviruses), assays of infectivity in coculture and TEM on ultrathin sections (U.S. Food and Drug Administration: http://www.fda.gov/cder/Guidance/Q5A-fnl.pdf; European Medicines Agency: http://www.emea.europa.eu/pdfs/human/ich/029595en.pdf) — for the detection and characterisation of retroviruses in the manufacturer's master and end-of-production cells (Figure 3). These agencies also recommend testing for viruses in unprocessed bulks (one or multiple pools of harvested cells and culture media). RT assays with such bulks are

hampered by high background levels due to cell-derived DNA polymerases and are no more sensitive overall than TEM with negative staining for retrovirus detection (Brorson et al., 2002). PCR assays are difficult to set up because investigators are faced with a multitude of murine viruses, many of which remain uncharacterised. Thus, TEM with negative staining appears to be a useful technique for ensuring the biosafety of biological products in these conditions.

The study of virus/host cell interactions: a link between the past, the present and the future

TEM is the only technique able to deliver clear images of viruses, due to their small size. Moreover, the fine detail of viral structure may become visible if viral preparations are rapidly frozen and the vitrified specimens examined by cryo-EM. When combined with data from X-ray diffraction studies, or with electron tomography or single-particle analyses of isolated virions, highly detailed structures can be obtained at near atomic resolution (Grunewald et al., 2003; Cyrklaff et al., 2005; Forster et al., 2005; Briggs et al., 2006; Harris et al., 2006; Roux and Taylor 2007). However, these approaches based on purified viral preparations are beyond the scope of this review, which focuses on the detection of viruses in fluids or infected cells or tissues. As obligate intracellular parasites, viruses depend on living cells for their replication. Interactions with host cells begin with the binding of the virus to specific receptors on the cell surface (Marsh and Helenius, 2006). Some viruses release their genomes directly into the cell by rupturing the plasma membrane, but most enter cells by endocytosis. Following their internalisation, viral particles are sequestered in endocytotic organelles until appropriate conditions for viral genome release into the cytoplasm occur. Two main sequestration strategies are used: enveloped viruses fuse with a cell membrane, whereas non-enveloped viruses partially disrupt cellular membranes. Further uncoating reactions and/or transport of the core may also be required. The viral genome is then translocated to specific sites in the cytoplasm or nucleus for replication and expression. The formation of "factories" has been reported for many viruses. These factories consist of perinuclear or cytoplasmic foci — mostly excluding host proteins and organelles but sometimes recruiting specific cell organelles — that form a unique structure characterised by a number of complex interactions and signalling events between cellular and viral factors (Wileman, Science 2006). The newly synthesised viral proteins and genetic material are then assembled into progeny viruses. Enveloped viruses acquire a lipid membrane by budding through a cellular membrane. Virus assembly may occur at the plasma membrane, but many viruses begin their assembly process in intracellular organelles, such as the endoplasmic reticulum, Golgi apparatus or endosomes (Griffiths and Rottier, 1982). Our understanding of the various stages of the viral life cycle has therefore been greatly enhanced by TEM studies, some of which have included immunolabelling protocols. For example, virus entry pathways based on the use of clathrin-coated pits or caveolae have been documented by TEM analysis for various viruses (Helenius et al., 1980; Kartenbeck et al., 1989; Bousarghin et al., 2003). TEM has been used to study the generation of new virions, providing particularly striking images of "viral factories" for several virus families (Novoa et al., 2005) (Figure 4). TEM has also proved important for the characterisation of morphological features at various stages in the assembly of viral particles, including the acquisition of lipid membranes by enveloped viruses, and for distinguishing between immature and mature particles (Hourioux et al., 2000; Pelchen-Matthews and Marsh 2007) (Figure 5).

However, the recent development of new imaging techniques for light microscopy has progressively limited the use of TEM for studies of virus/cell interactions. Viruses labelled with small fluorescent proteins or small dye molecules are currently the most powerful tools for studying dynamic interactions between viruses and the cellular machinery (reviewed recently by Brandenburg and Zhuang 2007). It is now possible to follow the trafficking of a single virus in a single cell. One of the chief disadvantages of TEM is the need to use dead and fixed samples. The new techniques, which can be used on living cells, make it possible to follow the fate of individual viral particles and to monitor dynamic interactions between viruses and cellular structures, making it possible to study steps in infection that were previously unobservable. For example, the long-standing debate concerning whether poliovirus breaches the plasma membrane barrier or relies on endocytosis to deliver its genome into cells was recently settled using this approach (Brandenburg et al. 2007). This study demonstrated that poliovirus enters cells via clathrin-, caveolin- and microtubule-independent but tyrosine kinase- and actin-dependent endocytosis. Many studies have also demonstrated that various viruses hijack the microtubule- and actin filament-based transport machinery for their transport within living cells (Brandenburg and Zhuang 2007).

Nevertheless, these fluorescence methods can reveal little about structure. Viruses can be visualised as small spots on fluorescence microscopy, but the resolution of this technique is too low to determine whether these fluorescent spots correspond to assembled virions or aggregated viral proteins. Thus, TEM remains an essential technique for visualising structured virions in infected cells, as illustrated by several recent studies. Hepatitis C virus (HCV) is unusual in that it was first identified by the cloning of its genome, before TEM visualisation (Roingeard et al., 2004). Even today, little is known about the morphogenesis of this virus. TEM on a virus-like particle (VLP) model obtained by expressing genes encoding the HCV structural proteins has demonstrated that viral budding occurs at the ER membrane and that the HCV core protein drives this process (Blanchard et al., 2003). Fluorescence microscopy has shown that most of the HCV core protein is associated with the surface of lipid droplets (McLauchlan et al., 2003), and TEM has shown that viral budding occurs at the ER membrane, in the close vicinity of these lipid droplets (Ait-Goughoulte et al., 2006; Hourioux et al., 2007) (Figure 6). TEM has also shed light on the morphogenesis and intracellular trafficking of subviral envelope particles associated with the hepatitis B virus (HBV). According to an initial model based on

biochemical and fluorescence microscopy studies, the major HBV envelope protein forms dimers in the ER membrane before its transport, as transmembrane dimers in vesicles, to the ER-Golgi intermediate compartment (ERGIC), where its self-assembly leads to the morphogenesis of subviral envelope particles (Huovila et al., 1992). However, recent TEM studies have shown that HBV subviral particles self-assemble into filaments within the ER lumen (Patient et al., 2007). TEM has also shown that these filaments are packed into crystal-like structures for transport by ER-derived vesicles to the ERGIC, where they are unpacked and relaxed (Figure 7). HIV morphogenesis also provides a remarkable example of intensive research on virus/host cell interactions, for which the debate concerning the site of virus assembly remains unresolved. Studies in this field make use of TEM to determine the site of virus assembly, which may occur at the plasma membrane or in late endosomes, depending on the virus/host cell system (Grigorov et al., 2006; Jouvenet et al., 2006; Pelchen-Matthews and Marsh 2007; Welsch et al., 2007). Thus, light microscopy and TEM are not exclusive and rather complementary. One particularly interesting development is correlative light electron microscopy (CLEM), which combines ultrastructural and fluorescence visualisation (Frishknecht et al., 2006). The use of quantum dots (Giepmans et al., 2005) or small genetic tags, such as tetra-cysteine motifs (Gaietta et al., 2002; Lanman et al., 2007), which are readily visible on both TEM and light microscopy, is useful for such approaches. Other methods such as GRAB (GFP Recognition After Bleaching) use oxygen radicals generated during the GFP bleaching process to photooxidize diaminobenzidine into an electron-dense precipitate that can be visualized by EM (Grabenbauer et al., 2005).

In conclusion, TEM is clearly less important than it once was in the field of diagnostic virology, but this technique remains useful for the occasional identification of unknown agents during particular outbreaks. In this respect, it is a valuable technique for controlling the viral safety of biological products. For research, TEM is complementary to other investigative techniques for elucidating many aspects of the life-cycle of the virus in an infected cell, including viral assembly in particular, as ultrastructural analyses may be remarkably informative.

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Figure 1

Diagnosis of virus infections by examiation of ultrathin sections of human tissues or cells

(A) Parapoxvirus (Orf virus) infection on a human skin biopsy specimen. Multiple oval viral particles (arrow) consisting of a dense core surrounded by an envelope (high magnification in inset) are observed in an infected cell. The Orf virus is a parapoxivus that causes a common skin disease of sheep and goats and is occasionally transmitted to human. (B) Polyomavirus (BK virus) infection in cells pelleted from a urine sample taken from an organ transplant patient. The presence of a large number of viral particles leads to their arrangement into a crystal-like structure (high magnification in inset).
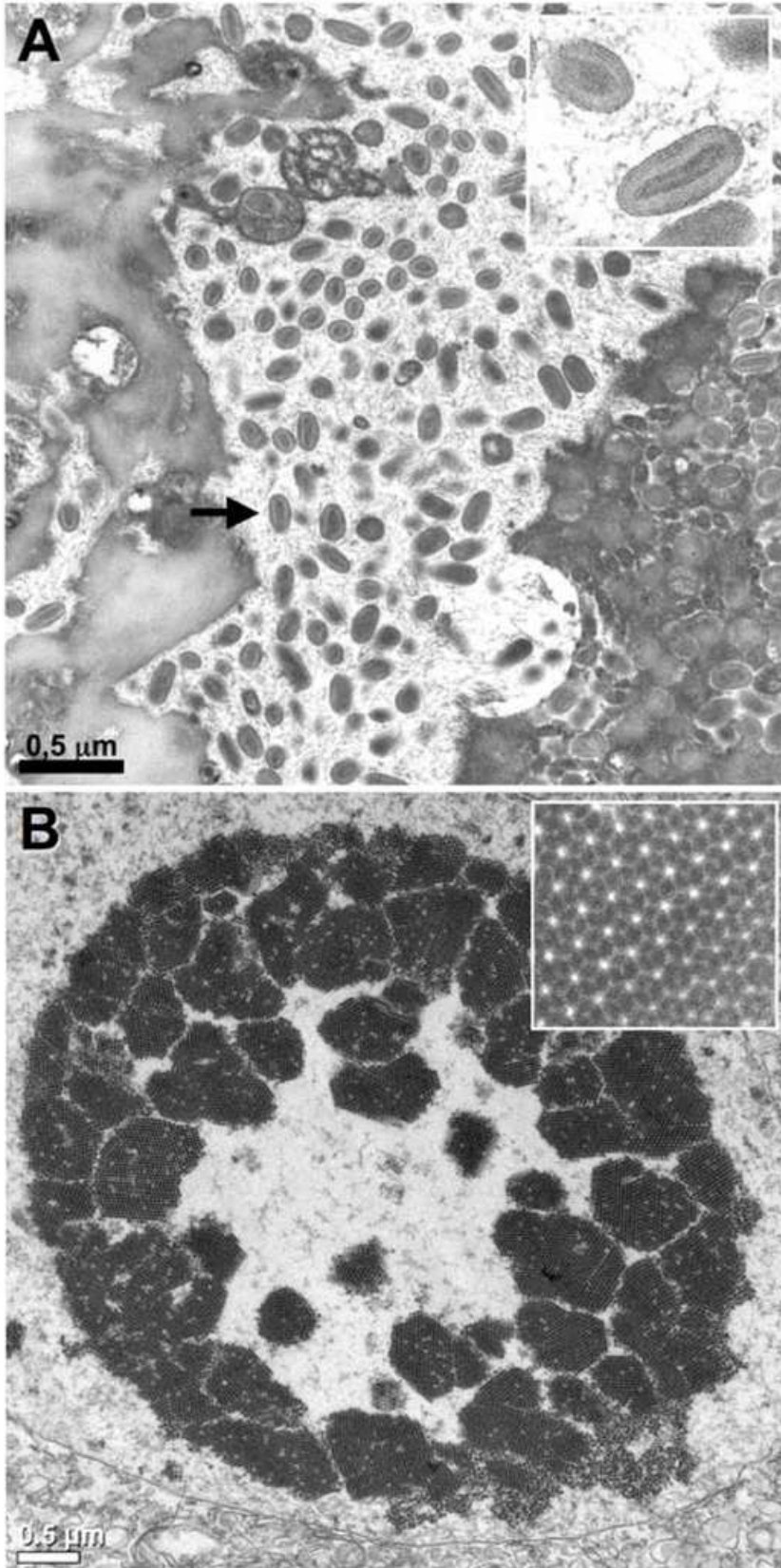
Figure 2
Direct negative staining of virus in fluid recovered from human skin vesicles (A and B) or from stool samples (C and D)
The panels A and B illustrate rapid morphological diagnosis and differential diagnosis from a herpesvirus (Varicella, in A) and a parapoxvirus (Orf virus, in B). The penetration of the negative stain into the herpesvirus particle may reveal the presence of the viral capsid within the envelope. The panels C and D show that a negative staining of viruses involved in gastroenteritis reveals the surface detail of the subunit arrangement of the adenovirus core particle (C), showing clearly its icosahedral form, whereas rotavirus displays its typical "wheel-like" appearance (D).
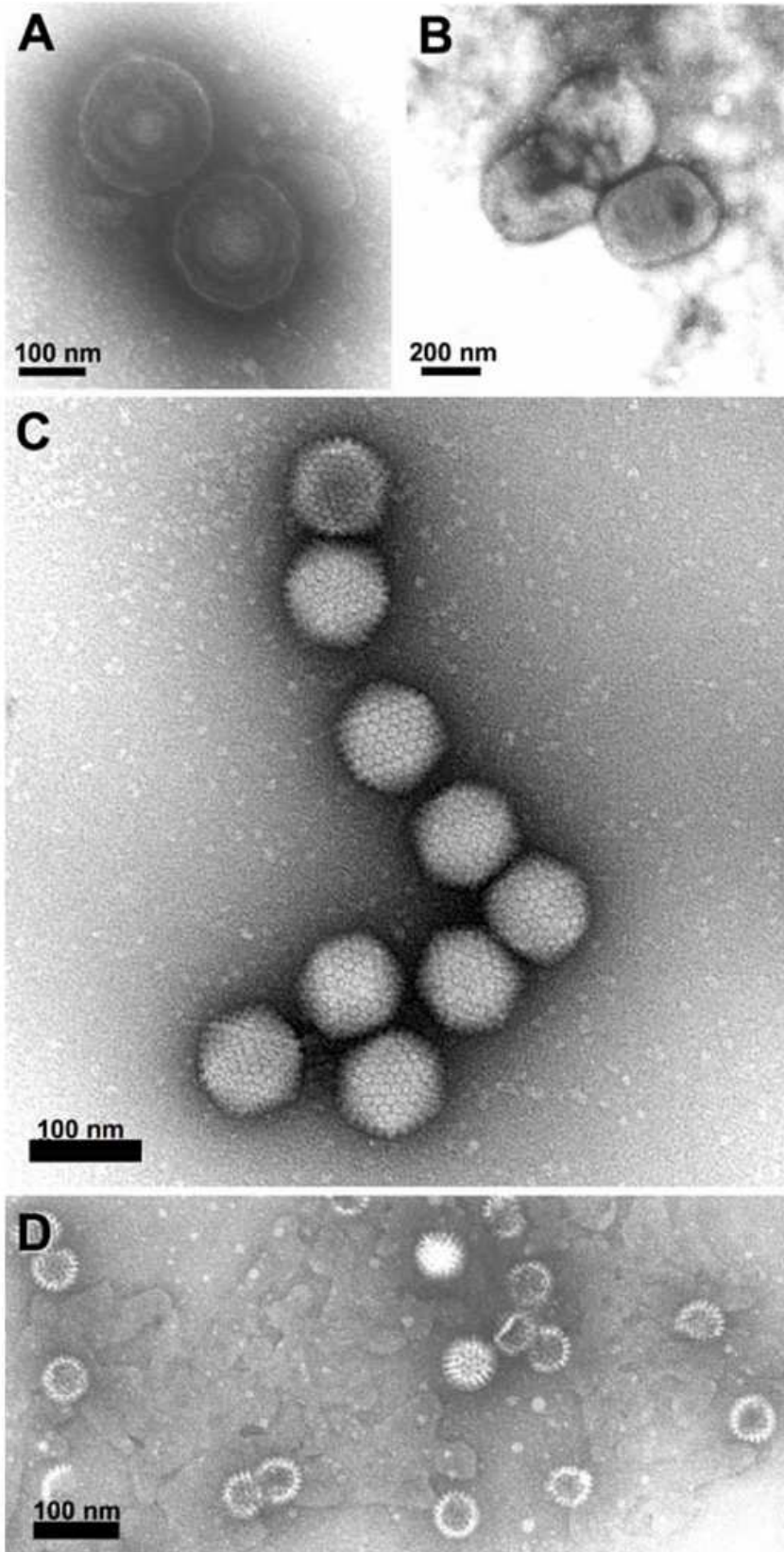
Figure 3
Detection of retroviruses in rodent hybridoma cells used for the production of biological products
Ultrathin sections of cells of different origins may show intracisternal A-type retroviral particles (in A) or C-type retroviral particles budding at the cell surface (in B). The C-type particles released by the cells can be detected by negative staining in the cell supernatant (inset in B).
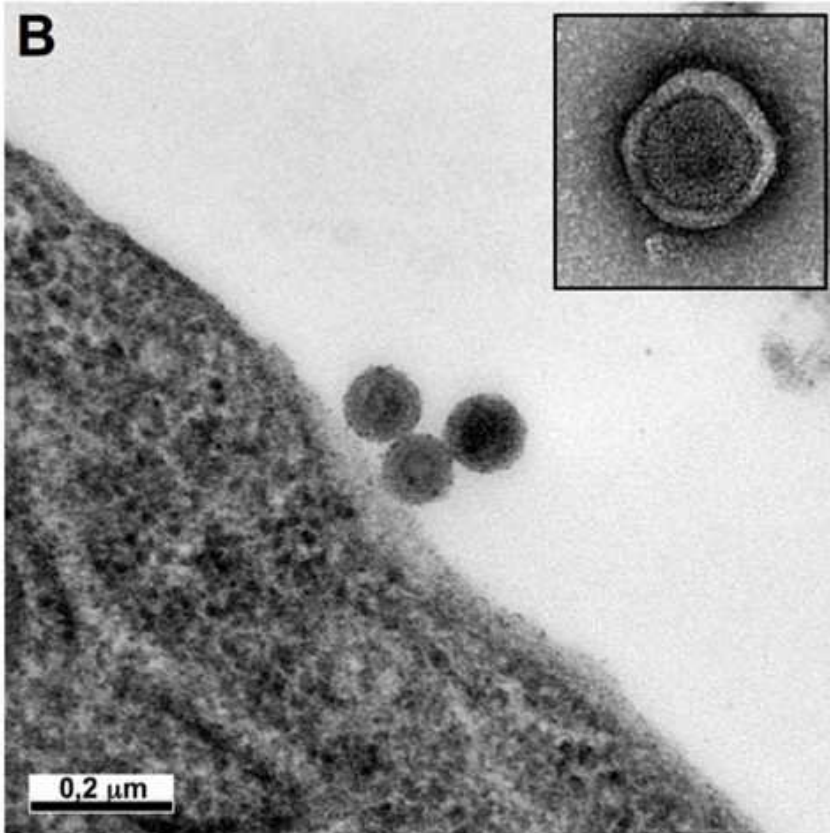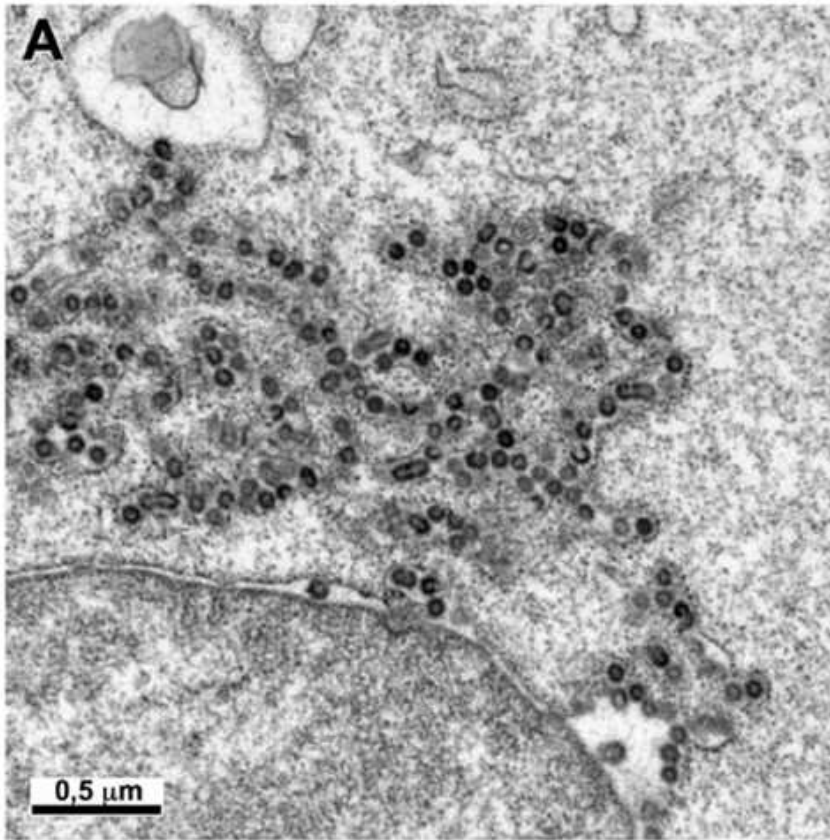
Figure 4

Ultrastructural changes associated with viral replication, or "viral factories"

A: the Semliki forest virus (SFV), an alphavirus, induces the formation of a cytopathic vacuole (CPV), surrounded by the endoplasmic reticulum (ER). Numerous viral replication complexes (arrow) are anchored in the internal membrane of these CPV. B: The non-structural proteins of the hepatitis C virus (HCV), a flavivirus, induce the formation of a membranous web in the perinuclear area.
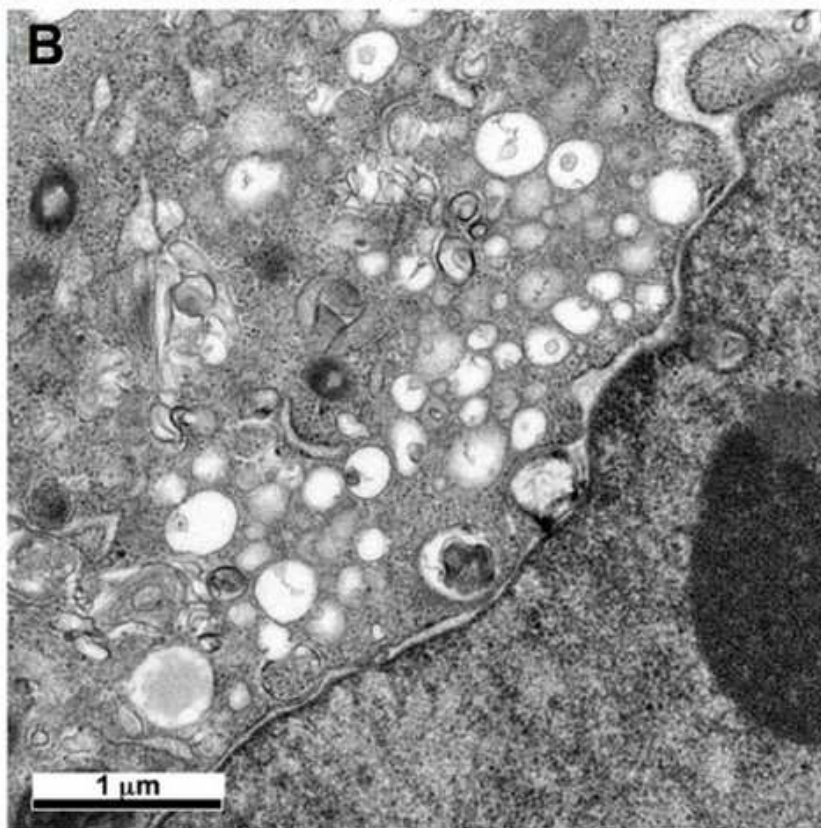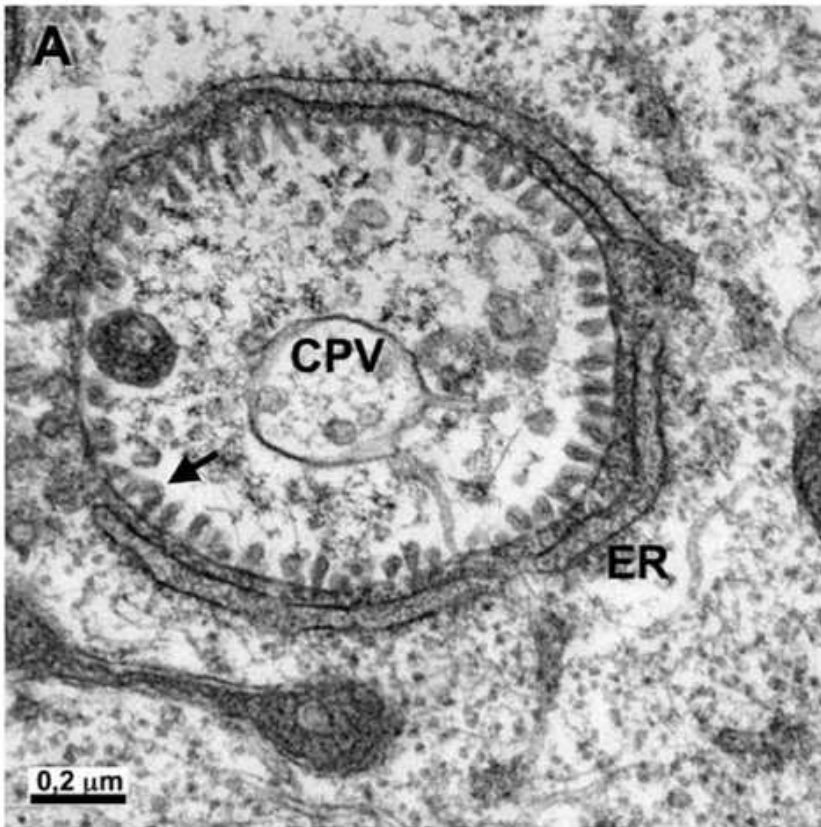
Figure 5

Budding of the human immunodeficiency virus (HIV)

The viral particle at the top shows virus formation with distortion of a cellular membrane away from the cytoplasm. The budding particle and the particle at the bottom are immature viral particles, whereas the two particles in the centre are mature, and have a truncated cone-shaped core. Thus, maturation of the core by the viral protease occurs shortly after the release of the particle from the host cell membrane.
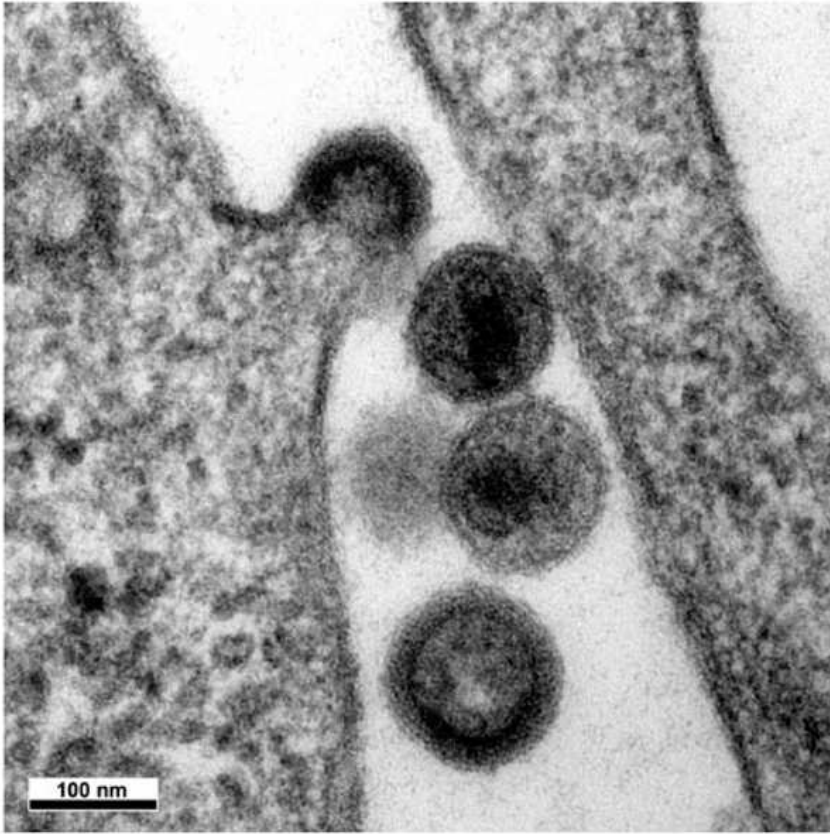
Figure 6
Budding of the hepatitis C virus (HCV)

Ultrastructural analysis of cells producing the HCV core protein shows that this protein self-assembles into HCV-like particles (arrows) at convoluted and electron-dense ER membranes surrounding the lipid droplets (LD) present in the perinuclear area.
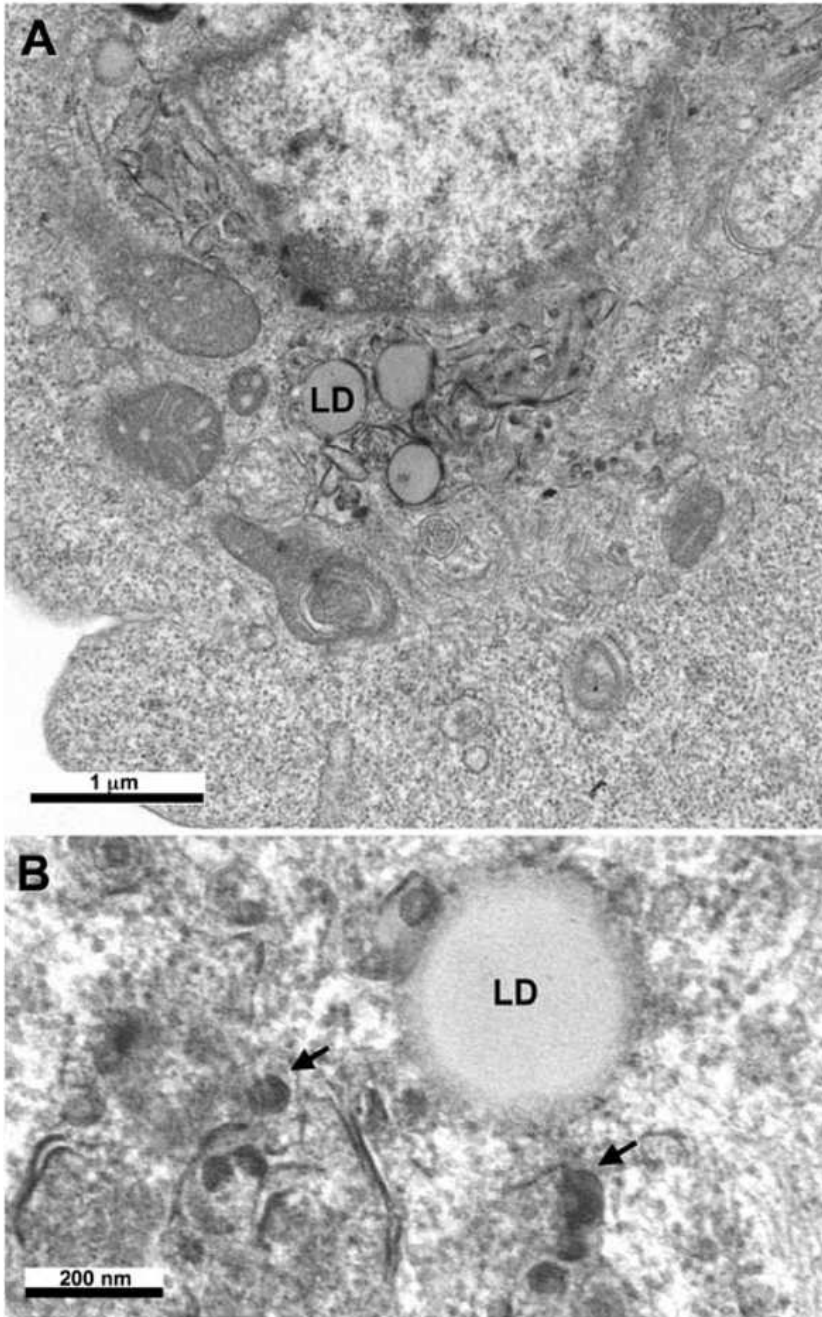
Figure 7

Hepatitis B subviral envelope particle morphogenesis and intracellular trafficking

Ultrastructural analysis of cells producing the hepatitis B virus (HBV) major envelope protein shows that this protein self-assembles in the ER into filaments packed into crystal-like structures (A, see also a high magnification of these packed filaments in the inset). These filaments are transported to the ERGIC, where they are unpacked and relaxed (B).