Comparative Ultrastructural Studies of Three Flaviviruses in Vero Cells

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Abstract: The complete details of the flaviviruses replication and maturation in Vero cells did prove to be elusive. However, from this study, a comparison was done at the ultrastructural level with the Kunjin, Japanese encephalitis (JE) and Murray Valley encephalitis (MVE) viruses-infected Vero cells. Except for one or two virus-induced membrane structures which was unique for either JE virus or MVE virus, on the whole the essential virus-induced structures were common among these three viruses. The electron miroscopic studies revealed numerous vesicles with thread-like enclosures within the distended cisternae of the endoplasmic reticulum. Convoluted smooth membraneous structures were also in close association with these vesicles and the rough endoplasmic reticulum. In sites where these three membrane components are present they are believed to be sites for virus condensation and perhaps maturation. Mature virus particles were often observed in clusters around these structures or within the distended lumen of endoplasmic reticulum. The release of the viruses were envisaged to be by reverse phagocytosis, via the channels of the endoplasmic reticulum and finally by cell lysis when the cytopathic effects became advanced at the late stages of infection. Attempts were made using immuno-gold protein A conjugated antibodies to try and locate some viral precursor products as the conventional electron microscopy was not able to portray. However, although specific binding of the antibodies did occur, the results obtained was not conclusive.

Key words : Flaviviruses, Ultrastructural change, Vero cells

INTRODUCTION

With the advent of improved techniques used to study the flaviviruses, these viruses are now reclassified and grouped under the new family of Flaviviridae (Westaway *et al.*,

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1985). Electron microscopic observations on the development of flaviviruses in verterbrate and invertebrate cells have led to the general conclusion that they mature within the cell in close association with the membranes of the endoplasmic reticulum and Golgi apparatus(Ota, 1965; Leary and Blair, 1980; Ng-in press). The mature virus particles are released from the cell by exocytosis or reverse phagocytosis. There has been to date no irrevocable evidence that flavivirurses develop and mature by budding through the plasma mambrane like the alphaviruses(Gliedman *et al.*, 1975).

It is the aim of this paper to present on the basis of electron microscopic evidence, the replication of three flaviviruses namely, Kunjin, Japanese encephalitis(JE)and Murray Valley encephalitis(MVE)viruses in Vero cells. Comparison was made on the similarities and differences between the replication of these viruses and their intracellular development. The possible roles of the cellular structures observed at the various stages of virus replication in infected cells will also be evaluated objectively.

MATERIALS AND METHODS

Viruses and cell cultures: The Nakayama strain of JE virus, MRM 66 strain of MVE virus and MRM 61C strain of Kunjin virus were used throughout. These viruses were obtained from Professor E. G. Westaway, Monash University, Australia. Vero cells were grown to confluency at 37℃ in Medium 199 containing 5% foetal calf serum and 0.1% DGP. They were maintained in Eagle's minimum essential medium (MEM) containing 0.1 % bovine serum albumin (BSA) after infection.

Electron microscopy: Cells were prefixed with 2.5% glutaraldehyde-2% paraformaldehyde mixture followed by post-fixation for one and a half hour at 4℃ in 1% osmium tetroxide. They were then dehydrated through a series of acetone of increasing concentration and embedded in low viscosity epoxy resin. Ultrathin sections (50nm−70nm) were stained with 2% uranyl acetate and post-stained with 2% lead citrate before viewing under a Philips electron microscope, 400T.

Immuno-electron microscopy (Immuno-gold labelling techique): *List and source of antibodies used.*

Antibodies raised against	*Dilution used*
anti-whole Kunjin virus	1:10
anti-Kunjin virus envelope	neat

Both the antibodies used were obtained as a gift from Professor E. G. Westaway, Monash University, Australia. These antisera were used for the immuno-fluorescent work published by Ng and co-workers(1983).

Procedure : The cells were rinsed in phosphate buffered saline(PBS)solution and then fixed at 4℃ with 1% paraformaldehyde-0.1% glutaraldehyde solution containing 0.002% saponin(Sigma, USA). The fixation was done for *exactly* 1 min at 4℃. The monolayer was then immediately washed to remove all traces of saponin. The monolayer was then refixed for 30 minutes at 4℃ with the same fixative as above but without saponin. At

the end of the 30 minutes fixation, the monolayer was again washed with cold PBS solution and the cells were scraped off with a rubber policeman and pelleted by low-speed centrifugation for 5 minutes.

The cell pellet was incubated overnight at 4℃ with 50 μl of specific antibody. On the following day, after low-speed centrifugation for 5 min, the supernatant was discarded and the pellet was washed with 1% Tween 20 in PBS solution for an hour. This washing step was repeated twice. Washing with 1% Tween-PBS solution prevents non-specific adsorption of antibodies (Ng *et al.*, 1985) . After these washings, the pelleted cells were incubated overnight with 50μl of diluted (1:20 parts with PBS solution) protein A-colloidal gold (pAg) particles (5 nm in diameter, Janssen Pharmaceutica, Belgium) at 4℃. On the following day, after low-speed centrifugation, the supernatant was discarded and the pellet of cells was washed twice with cold PBS solution at 4℃ for an hour each before the pellet of cells was fixed and processed as for transmission electron microscopy (see above).

RESULT

Replication of flaviviruses in Vero cells

All the three flaviviruses, namely, Kunjin, Japanese encephalitis (JE) and Murray Valley encephalitis (MVE) viruses caused cytopathic effects in infected Vero cells. The latent periods of these three viruses were relatively similar and were between 12−14 hours post infection (p. i.). Initial signs of cytopathic effects were noticeable at about 24 hours p. i. and by 30 hr p. i. a high proportion of the infected cells had rounded up. Eventually, the rounded cells would lift off the growing surface. Thus under phase contrast light microscopy, the general morphological changes of the cells infected with the three viruses are similar. It was then interesting to see if ultrastructural studies would be able to differentiate some specific virus-induced changes of each virus in Vero cells.

Ultrastructural studies of Kunjin, Japanese encephalitis and Murray Valley encephalitis viruses-infected Vero cells

The uninfected Vero cells displayed the normal range of organelles (Fig. 1a). During the latent periods of the three viruses, there was no obvious ultrastructural modification. However, 24 hours post infection with any of the three viruses, approximately 60% of the infected Vero cells showed an appreciable increase in the amount of smooth and rough endoplasmic reticulum (SER and RER respectively; Fig. 1b).

After 32 hours of infection with Kunjin virus, clusters of oval-shaped vesicles (60−80 nm in diameter) were found scattered in large clusters in the cytoplasm especially at the perinuclear region (Fig. 2a). Practically every vesicle had a distinct filament-like structure enclosed within it. It has been postulated that this filament-like structure might be the virus progeny RNA (Ng, in press). These vesicles were also often observed associated with the convoluted smooth membrane ('CM' − Fig. 2b). The smooth membrane seemed

in turn to be connected with the RER. On careful examination, the lumen of the rough endoplasmic reticulum were filled with morphologically mature virus particles (arrowheads, Fig. 2b). These sites could be the locations for the synthesis of virus precursors as well as virus maturation.

The appearance of these vesicles in both the JE and MVE viruses-infected cells were slightly delayed. The numbers became significant at about 48 hours p. i. These vesicles again contained obvious electron dense fibrils or center (Fig. 3a). Many of the vesicles also appeared to be in close association with the RER; in fact, rows of vesicles were seen enclosed within the thickened membranes of the endoplasmic reticulum (Fig. 3a and b). At this time electron-dense masses were frequently observed in the infected cytoplasm (Fig. 3c). These dense areas could be very compact structures of the 'CM' as seen in the Kunjin virus infection (see Fig. 2b). The RER and vesicles were similarly observed to be in close association with these dense smooth membranes. A cluster of virus particles was seen in close vicinity with the vesicles.

A virus-induced membrane structure which was unique to the JE virus-infected cells was the clusters of large irregular-shaped membraneous structures (LMS—Fig. 4a). The 'LMS' was observed in addition to the vesicles and they differed from the smaller uniform-sized vesicles in that their sizes and shapes were larger and variable. In the MVE virus-infected cells, another type of virus-induced membrane structure was seen in place of the 'LMS'. These were the tubular structures (TS—Fig. 4b). Again these structures were present in addition to the uniform-sized vesicles. When viewed in cross sections, they appeared to be thick-walled and occasionally the lumen also contained 'eccentric' granular enclosures. Some of the 'TS' were seen enclosed within the extremely distended lamella of the RER. Mature virus in small clusters lie close to the 'TS' and RER.

By 54 hours p. i. (for the three viruses), extensive proliferation and hypertrophy of the SER was observed (not shown) filling up a large volume of the cytoplasmic space. The wall of the SER appeared thickened but agranular. The lumen were distended to accomodate the morphologically mature virus particles as well as the vesicles (Fig. 4c).

Despite some minor differences in virus-induced membrane structures among the three viruses-infected cells at the earlier stages of infection, by 72 hours p. i., the ultrastructural morphologies were again similar among the three viruses. By this stage of the infection, large numbers of virus particles had accumulated intra- and extracellularly (Fig. 5). Several possible modes of virus release could be envisaged. Mature virus particles were often seen in large vacuoles very close to the periphery of the cells (Fig. 5a). It is possible that the virus particles could be released through the process of reverse phagocytosis i. e. by fusion of the vacuolar membrane with the plasma membrane. Another alternative mode could be via the lengthened endoplasmic reticulum forming long channels. Occasionally, these channels have been seen to lead to an opening at the plasma membrane (Fig. 5b). As the cytopathic effects progressed, cell lysis would be an important factor in liberating the mature virus particles (Fig. 5c).

Immuno-electron microscopy

Since the conventional electron microscopy procedure described above did not enable us to detect any intermediate stages of the flavivirus morphogenesis or any viral precursor products, the technique of immuno-electron microscopy using protein A-colloidal gold was attempted. Polyclonal antibodies were used in this preliminary study rather than monoclonal, to facilitate easier location of viral precursor antigens within the cell. Only Kunjin virus infected cells were studied as the antisera were readily available.

(a) *Antibodies raised against the whole virus*

When mock-infected cells were processed similarly against anti-Kunjin virus serum, no non-specific binding of gold particles was observed (Fig. 6a). However, with infected cells, in areas where morphologically mature virus particles were present, gold particles were seen adsorbed onto exposed viruses but not on virus particles present within the channels (Fig. 6b). The gold particles were also observed to be clustering in areas of the cytoplasm close to the virus-filled channels. The gold-conjugated antibody did not appear to be able to penetrate the smooth membrane channels containing the virus particles (Fig. 6b). Also where the virus lattices were large, the gold particles seemed unable to penetrate readily into these lattices. However, exposed virus particles were often surrounded specifically with the labelled antibody (Fig. 6c).

(b) *Antibodies raised against the envelope of the Kunjin virus*

Since the anti-whole virus serum did not attach to every virus particle, a more specific antiserum was used. This was the gold-conjugated antibody against the envelope component of Kunjin virus. It was hoped that by following the locations of the gold particles within infected cells we would be able to obtain preliminary evidence for the possible site(s) of synthesis or accumulation of the virus envelope. However, complete success is still elusive although we did observe some specific binding (Fig. 7).

As a control, an infected culture was treated with the protein A-colloidal gold but without any anti-serum. There was a complete absence of gold in the infected cells (Fig. 7a). This indicated that there was no non-specific binding of the gold particles in these infected cells.

In the infected cells which were treated with anti-envelope serum, specific association of gold particles appeared to be attached onto virus particles even when the virus particles were within the smooth membrane channels (Figs. 7b and c). Unfortunately, the gold particles did not attach onto any specific sites or onto any of the virus-induced structures which were previously described above.

DISCUSSION

Even though the detail sequences of morphogenesis were not elucidated from this study, the progressive changes in the infected cells' ultrastructures were recorded.

The first obvious ultrastructural change was the extensive rearrangement and proliferation of the endoplasmic reticulum (ER) after the latent period in the infected cells (Fig. 1b) which increased in complexity as the infection advanced. This concurred with the reported works of Ko and colleagues(1979), Westaway and Ng(1980) and Murphy(1980).

The pronounced appearance of these cellular structures can be correlated to the various stages in the replication of the virus. The lengthening of the rough endoplasmic reticulum (RER—Fig. 3b) for example, can be related to rapid viral protein synthesis. An important role of the smooth endoplasmic reticulum (SER) is in the metabolism of lipids. Lipids, besides being an important structural component of enveloped viruses is also needed for the transport of certain viral components to the actual site of virus assembly (Blough and Tiffany, 1973, 1975). The role of the SER is thus envisaged to be that of lipid metabolism early in infection for the initial transport of viral components and later in infection for the formation of glycolipids, part of the structural component of the envelope.

During infection, the Golgi apparatus and its associated vesicles also increased in size and number especially for the JE and MVE viruses-infected cells. Virus particles were, however, not seen within the Golgi apparatus at any time during the infection contrary to the reports by Leary and Blair (1980). However, there is little cause to doubt that the Golgi apparatus is largely responsible for the end addition of carbohydrates to the envelope protein of JE and MVE viruses (Shapiro *et al.*, 1973; Wright, 1982). It appears therefore that the proliferation of cellular organelles, assuming that their original functions have not been modified, play important roles at the various crucial virus replication stages.

Structures not seen in uninfected cells, namely, the vesicles (Ve) or smooth membraneous structures (Calberg-Bacq *et al.*, 1975), convoluted membraneous structures ('CM' Leary and Blair, 1980), the tubular structures (Murphy, 1980) and the large membraneous structures (LMS) were also observed in the infected cells. The oval-shaped smooth vesicles with the thread-like enclosures (Figs. 2 and 3) have generated great interest. Leary and Blair(1980) proposed that these vesicles bud from the membranes of the RER to become localised within the RER and contained capsid proteins and viral RNA genome. They then condense, detach from the RER and are transported to the Golgi apparatus where glycosylation and maturation occurs.

However, no evidence of vesicles budding from the RER were seen in this study although large clusters of vesicles were often observed in close association with the RER and SER. It is probable that these vesicles carry the progeny viral RNA to the sites of protein (RER) and lipid (SER) synthesis. The sites where these three structural components are present are the probable locations where virus maturation occurs.

The tubular structures (TS—Fig. 4b) usually appearing as thick-walled spherical or elongated structures (depending on the plane of sectioning) were also observed by Murphy(1980) in BHK-21 cells infected with St. Louis encephalitis virus. Their role in the

replication of the viruses may be similar to the large membraneous structures (LMS—Fig. 4a) of JE virus-infected Vero cells. However their functions are currently unknown.

Since conventional transmission electron microscopy did not reveal the presence of any viral intermediates during the replication cycle in Vero cells, it was decided that a more precise method like immuno-electron microscopy procedure could assist. When colloidal gold was conjugated to antibodies against the whole virus, gold particles were seen only around the exposed, mature virus particles (Figs. 6b and c). No gold particles could be seen around virus that were enclosed within the smooth membrane channels or within the virus lattice. This could probably be due to the permeability barrier of the smooth membranes of the channels enclosing the virus particles. Even though using anti-envelope serum did seem to be more specific as gold particles can be seen attached to virus particles within the channels (Figs. 7b and c), the results were not satisfactory. This technique of immuno-electron microscopy may have great potential but the details of the procedure require further investigation before useful information can be readily obtained from it.

The key element of this study is not to elucidate the elusive process of flaviviruses replication. Instead, the timely appearance of the various virus-induced cellular organelles and viral structures at the various stages post-infection were studied in relation to the possible roles they might play among the flaviviruses. There is very little doubt that they are essential to the development and maturation of the virus. As there is no evidence of virus release through the plasma membrane, maturation is probably completed intracellularly. Release of the virus is most likely by exocytosis, transport via the channels of the endoplasmic reticulum or by cell lysis (late in infection).

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VERO 細胞を用いた3種のフラビウイルスの超微細構造学的研究

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フラビウイルスの VERO 細胞における増殖と成熟課程の詳細は未だ完全には理解されていない。この研究では Kunjin, Murray Valley 脳炎 (MVE), 日本脳炎 (JE)といった3種のフラビウイルスに感染した VERO 細胞における超微細構造学的変化を比較した. JE 又は MVE に特徴的なウイルスによる感染による1, 2の膜構造の変化を除いては, 全体的にこの3種のウイルスによる構造変化には共通性がある. 電子顕微鏡的に, 拡大した小胞体の内腔に糸様構造物を含む多数の空胞が見られ, 旋回した平滑な膜様構造がこられの空胞と粗面小胞体に隣接して存在する. これら3種の膜様構造物が存在する所で多分ウイルスの濃縮と成熟が起こると考えられる. これらの構造物の周囲とか拡大した小胞体内腔にしばしば成熟ウイルス粒子が集合している. ウイルスの放出は小胞体系を通ずる逆呑食作用あるいは感染後期に細胞病変が進行した場合には細胞の溶解によって起こると考えられる. 金の微細粒子を免疫学的に結合したプロテインAと抗体とを反応させる事によって通常の電子顕微鏡では確認出来ないウイルスの前駆体の存在場所を明らかにしようとしたが, その結果は必ずしも明確なものではなかった.

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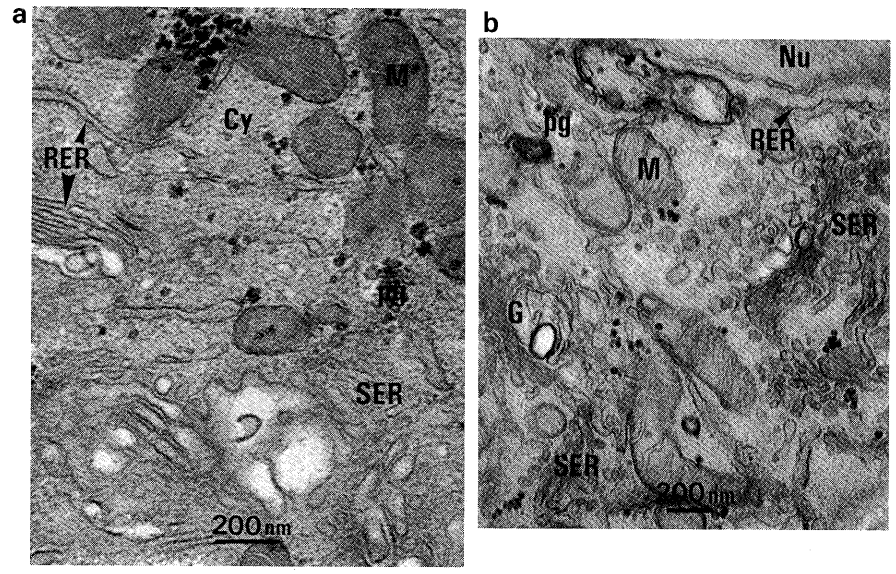

Fig. 1.
(a) Electron micrograph of unifected Vero cells. All the normal range of cell organelles can be seen.
(b) Electron micrograph of flaviviruses-infected Vero cells at 24 hours p. i. Proliferation and distensions of the smooth endoplasmic reticulum (SER) are apparent. The letterings represent : Nu-nucleus, Cy-cytoplasm, M-mitochondria, RER-rough endoplasmic reticulum, SER-smooth endoplasmic reticulum, pg-pools of glycogen and G-Golgi apparatus.

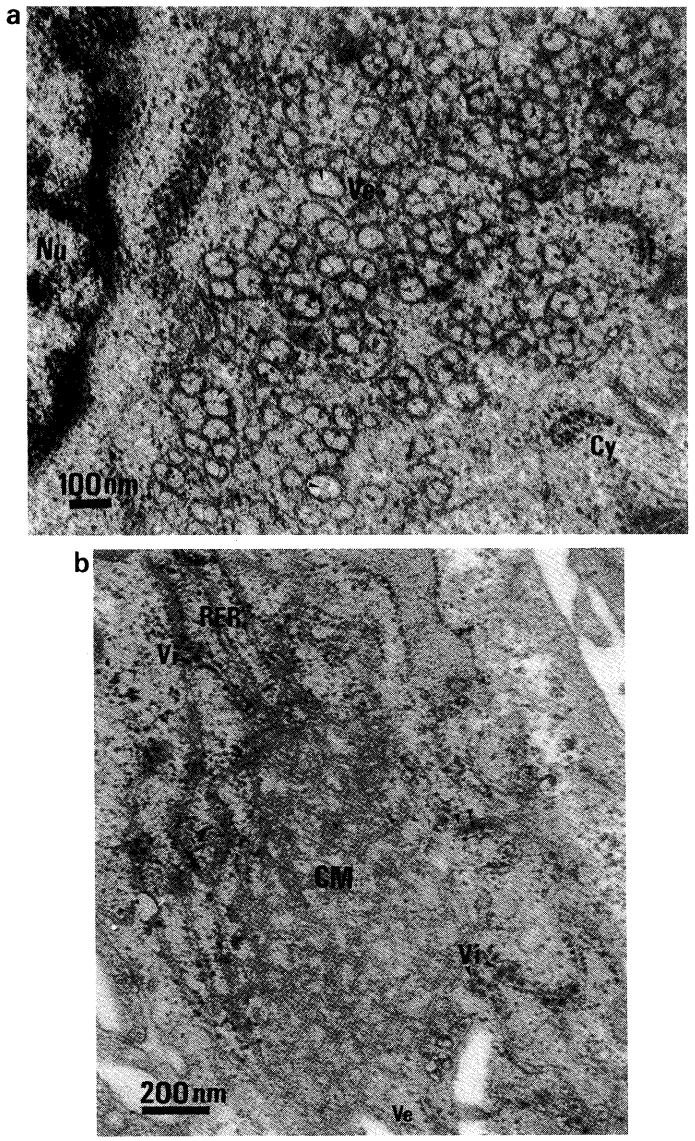

Fig. 2.
Kunjin virus-infected Vero cells at 32 hours p. i.
(a) Note the large aggregation of vesicles (Ve) at the perinuclear region. The filament-like structure in the vesicles are indicated by arrow-heads. The letterings represent : Nu-nucleus and Cy-cytoplasm.
(b) The convoluted smooth membrane (CM) is found in close proximity and connected with the rough endoplasmic reticulum (RER). Mature virus particles (Vi) are found within the distended lumen of the RER (indicated by arrow-heads). Vesicle (Ve) are also seen in the vicinity of these membrane structures.

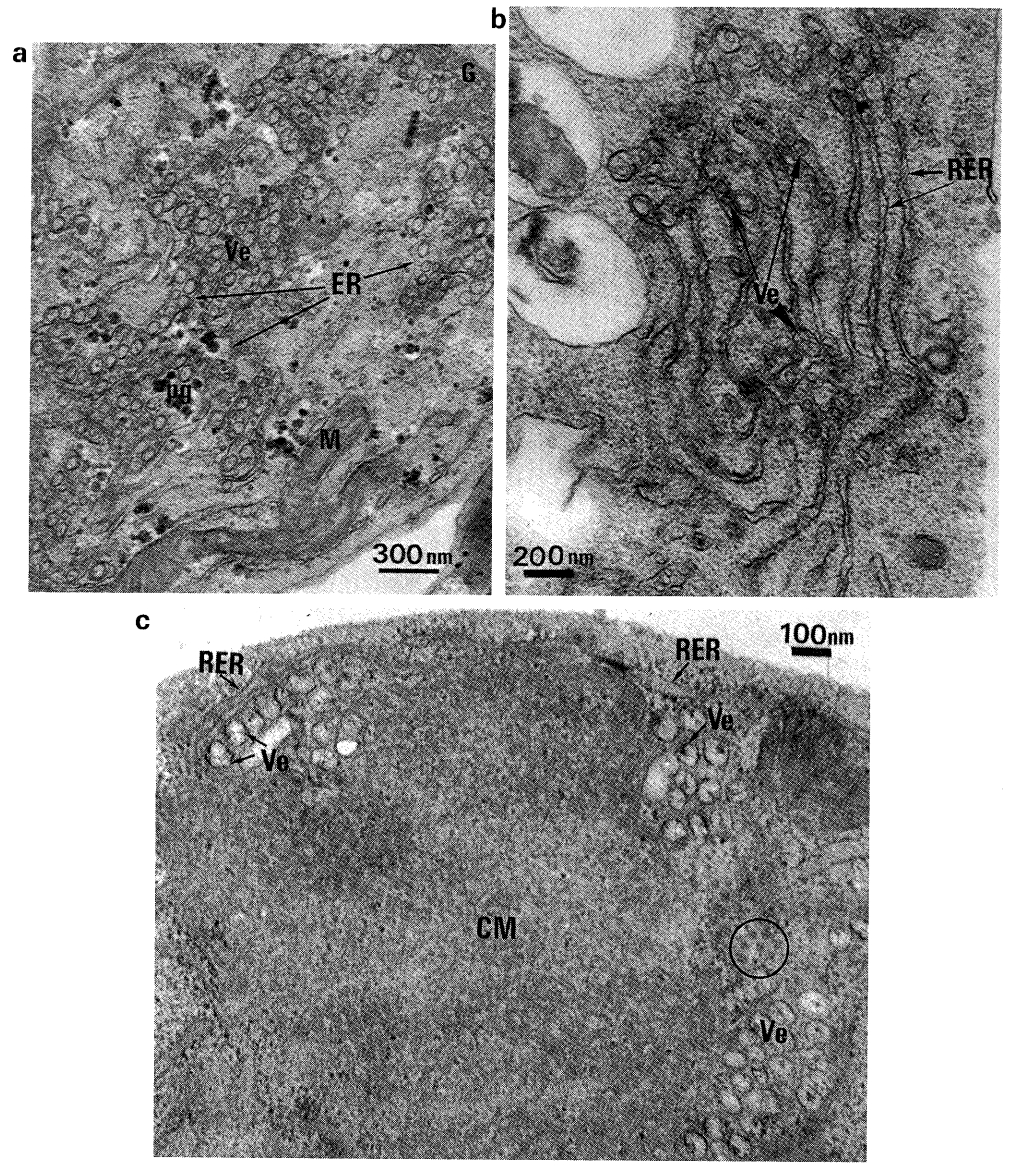

Fig. 3.
JE and MVE viruses-infected Vero cells at 48 hours p. i.
(a) The vesicles seemed to aggregate within the distended cisternae of the endoplasmic reticulum (ER). Majority of the vesicles contained thread-like enclosures. The Golgi apparatus (G) is found in close juxtaposition to clusters of vesicles. The letterings represent : pg-pools of glycogen and M-mitochondria.
(b) The extent of lengthening of the RER is clearly shown. Some sections of the distended RER lumen contain the vesicles (Ve).
(c) The extensiveness of the electron dense area can be seen in close association with the RER. This dense area could be the compact form of the 'CM' as seen in Figure 2b. Vesicles (Ve) are seen clearly associated with this dense membrane structure. Cluster of virus particles (circled) is also in close vicinity with the RER and the dense convoluted smooth membrane structure (CM).

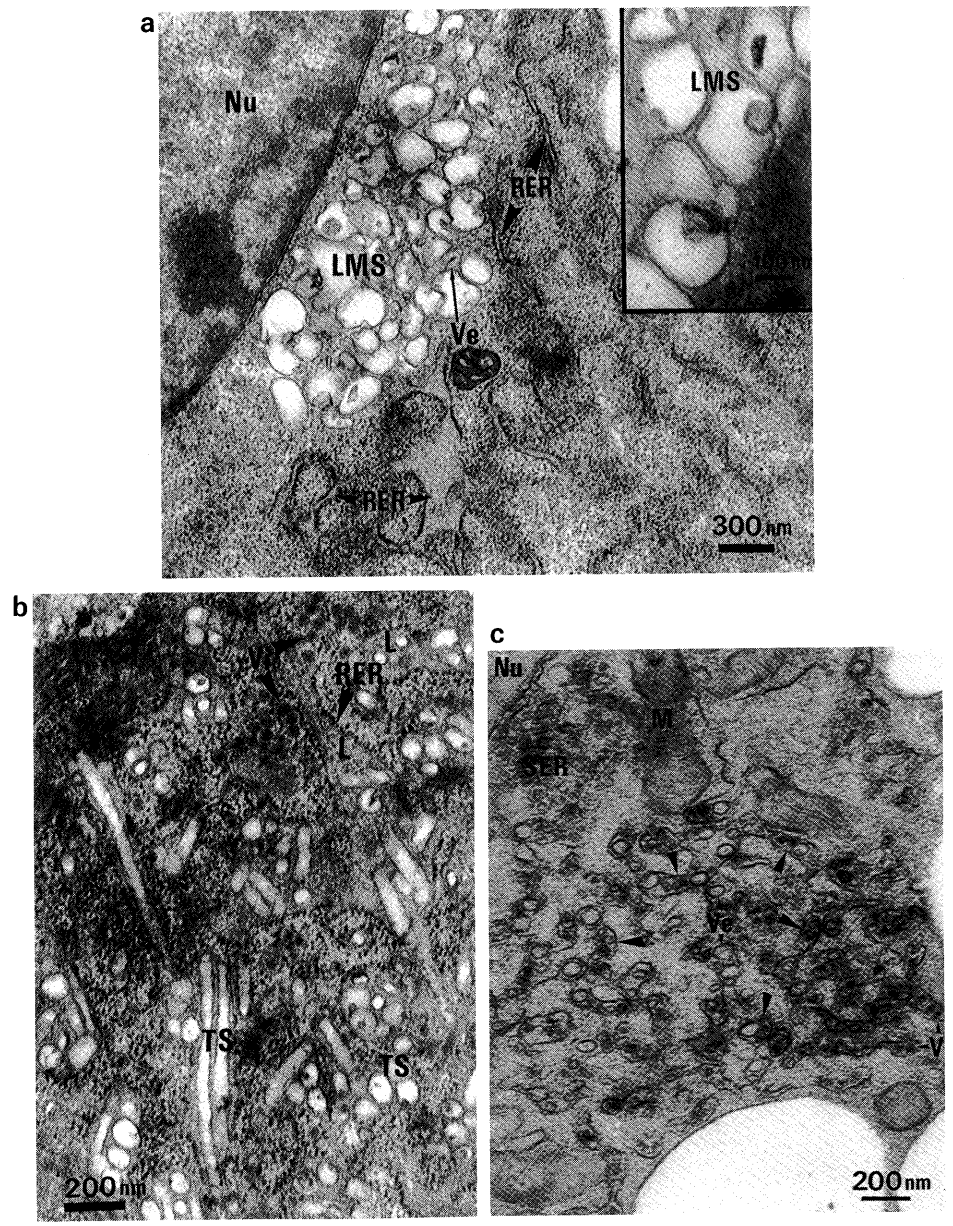

Fig. 4
(a) JE virus-infected Vero cells. Large irregular membraneous structures (LMS), some with an electron-dense eccentric granule lie close to the nucleus (Nu). The rough endoplasmic reticulum (RER) are seen scattered throughout the cytoplasm, seemingly without pattern and continuity. The inset showed the bilayered membranes of the 'LMS'.
(b) MVE virus-infected Vero cells. Numerous tubular structures (TS), many seen in cross sections are observed within the very much distended lamella (L) of the RER. The lumen of the RER also contained clusters of virus particles (Vi).
(c) 54 hours post infection for the three viruses. Mature virus particles (Vi) are frequently observed in association with the vesicles (Ve) within the distended lumen of the endoplasmic reticulum (arrow heads). The letterings represent : Nu-Nucleus, M-Mitochondria and SER-smooth endoplasmic reticulum.

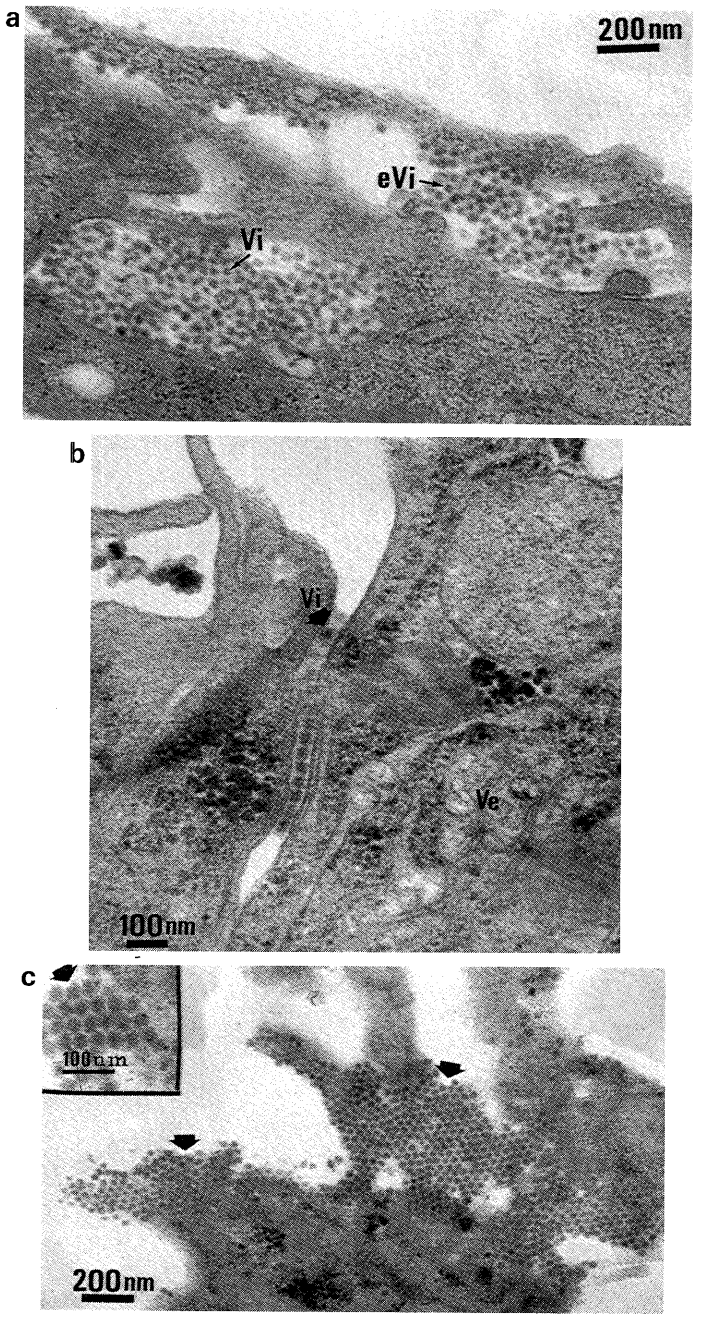

Fig. 5.
Flaviviruses-infected Vero cells at 72 hr p. i. All the three viruses used in this study gave the same ultrastructural morphology at this stage of infection.
(a) Mature virus particles (Vi) are seen here enclosed within large vacuoles and also in the extracellular space (eVi).
(b) Virus particles are also observed lining neatly within the lumen of the endoplasmic reticulum and occasionally these channels filled with virus can be seen leading out of the cells. The letterings represent : Vi-virus and Ve-vesicles.
(c) The arrows indicate a large accumulation of extracellular virus particles in the lattice formation. The inset shows a higher magnification of a virus lattice.

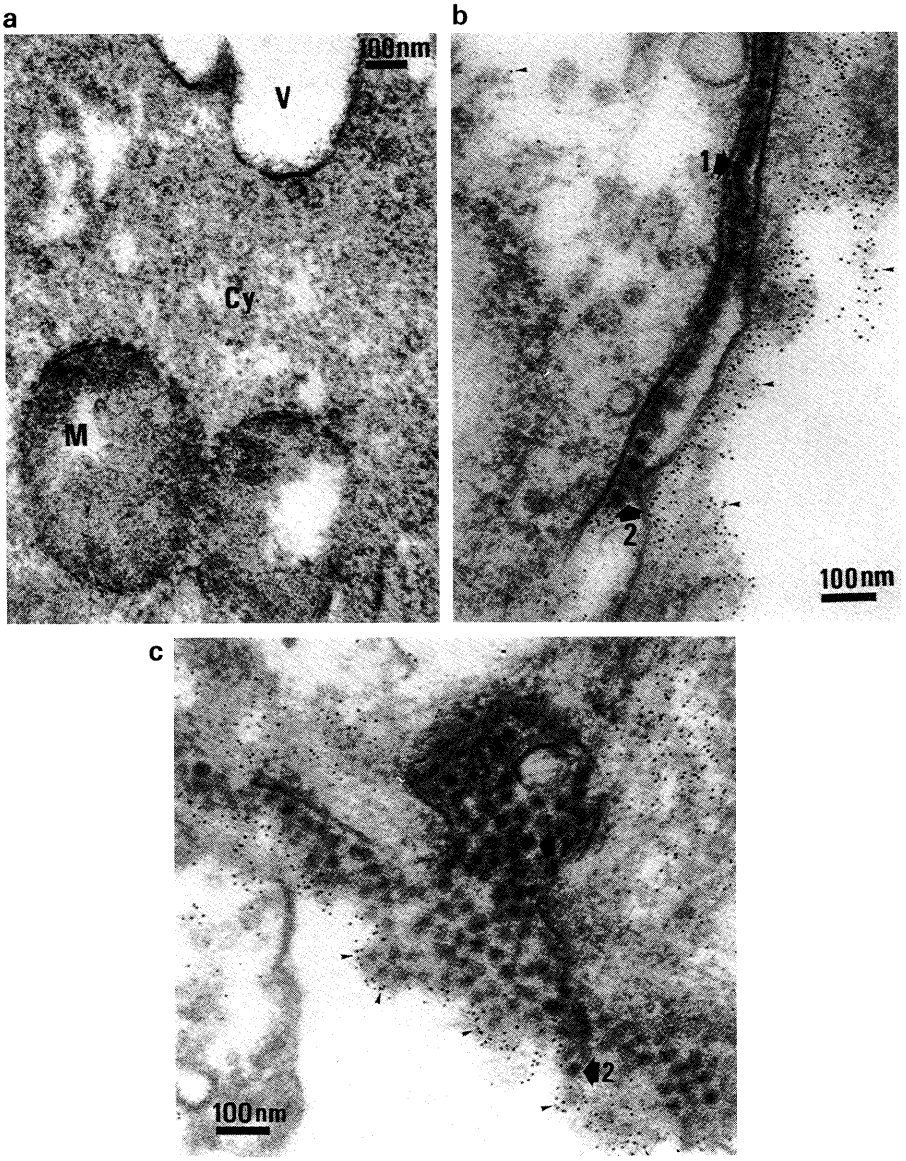

Fig. 6.
Uninfected and Kunjin virus-infected Vero cells incubated with Protein A-colloidal gold (pAg−5 nm) conjugated with antibodies directed against the whole virus.
(a) Uninfected Vero cells. There is an absence of gold particles adhesion within the cytoplasm (Cy). No non-specific binding of the gold particles can be seen within cytoplasmic organelles for example in the mitochondria (M) and vacuoles (V).
(b) In the infected cells, it is observed that the adsorption of the gold particles is only confined to the exposed virus particles (arrow 2) but not to the unexposed virus particles within the channels (indicated by arrow 1). The arrow-heads at the edge of the infected cells illustrate the presence of gold particles.
(c) Again, the specific adsorption of gold particles onto exposed virus particles (indicated by arrow 2 and the arrow-heads) is seen. No gold particles are seen adsorbed onto virus particles within the lattice (arrow 1).

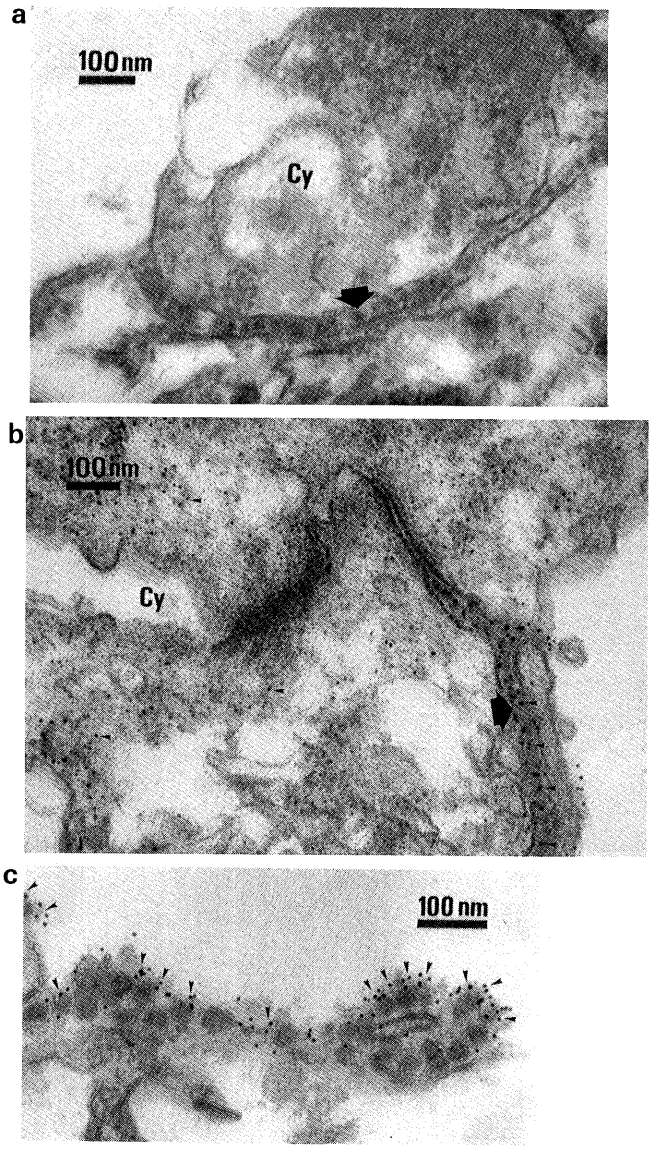

Fig. 7.
Kunjin virus infected-Vero cells incubated with Protein A-colloidal gold (pAg−5 nm) conjugated with antibodies against the Kunjin virus envelope protein.
(a) Infected cells not treated with antiserum. No gold particles can be seen in the cells indicating that the gold particles adhere only in the presence of antibodies. The arrow indicates a single row of virus particles enclosed within a channel. Cy-cytoplasm.
(b) Infected cells treated with antiserum. There is specific adsorption of gold particles onto virus particles. Specific adsorption is also noted with virus particles enclosed within the channel (indicated by the arrow). The arrow-heads indicate the gold particles.
(c) A higher magnification of virus particles in the channel. Again the arrow-heads indicate the gold particles on the virus particles.