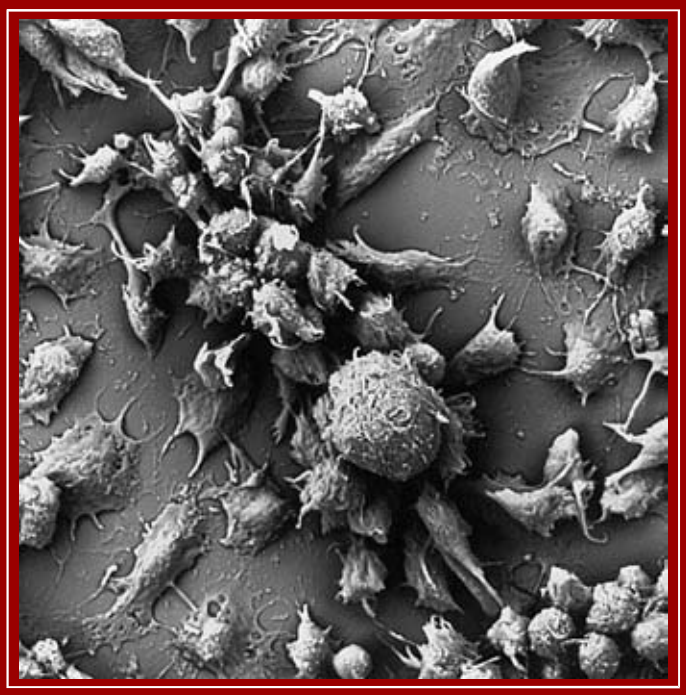
ATLAS Of Dengue Viruses Morphology And Morphogenesis



ORTRUD MONIKA BARTH

Ministry of Health/Brazil

Oswaldo Cruz Foundation/FIOCRUZ
Instituto Oswaldo Cruz

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FIRST CENTENARY: 1900 - 2000

ORTRUD MONIKA BARTH

ATLAS OF DENGUE VIRUSES MORPHOLOGY AND MORPHOGENESIS

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AUTHOR BIOGRAPHY

Ortrud Monika Barth

A native of Germany, Ortrud Monika Barth grew up in Rio de Janeiro, where she got her Ph.D. degree at the Department of Botany of the Federal University of Rio de Janeiro in 1964. A post-doctor fellowship followed in the Universities of Heidelberg and Freiburg in Germany. Pollen grain morphology and its application in science, as well as ultrastructural research in Biology, introduced she as a student into the Instituto Oswaldo Cruz. There she built a research group in Palynology in the Department of Ecology, along twenty years. In 1979 she moved to the Department of Virology, for virus diagnosis and morphogenesis in electron microscopy, as Head of the Laboratory of Viral Ultrastructure.

For several years O. Monika Barth was a member of the direction of the Brazilian Society for Electron Microscopy, Professor for Palynology and is the Head of the Laboratories of Palynology in the Departments of Botany and Geology at the Federal University of Rio de Janeiro. Since 1962 she is a fellow of the National Research Council (CNPq).

She carried out collaborative research projects in Germany, England and France and as author and co-author published more than 300 scientific papers, five books, 15 chapters in books and has orientated many post-graduate students in both fields of specialization.

E-mail: barth@ioc.fiocruz.br

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To

Hermann Gonçalves Schatzmayr

and

Marguerite Scott Pereira

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FOREWORD

It is a great pleasure to introduce this Atlas of Dengue Viruses Morphology and Morphogenesis, which summarize the data obtained in the Viral Ultrastructure Laboratory of our Department of Virology by Dr. O.M. Barth, along the last ten years.

After presenting the virus and dengue infected cells in its general aspects, the morphogenesis of dengue viral replication is described and discussed, aiming to obtain clearer definition of the events involved in the replication.

The book combines skilfull technical aspects with scientific criticism and is intended to be used as a reference, to those in the field of dengue morphogenesis and replication, as well as to electron microscopists and virologists in general.

Dengue virus infections became the most important arthropod borne viral disease in the world, along the last two decades and every effort to better understand its replication and morphogenesis should be encouraged. We consider this book an excellent contribution in these aspects.

This publication is dedicated to the Instituto Oswaldo Cruz, in its Centenary accomplished by May 2000, pointing out its vast contribution for research in tropical diseases, including dengue and yellow fever.

Dr. Hermann G. Schatzmayr
Head, Virology Department
Instituto Oswaldo Cruz
Rio de Janeiro, Brasil

PREFACE

The replication process of viruses of the *Flaviridae* family is still not yet completely known. Many papers about this subject deal with dengue viruses and the yellow fever virus, both of the genus *Flavivirus.* Beside these viruses, the *Flaviviridae* family comprises others RNA viral members, which morphogenesis, viral particles assembly and cell release, as well as persistent cell infections constitute a scientific challenge (Barth, 1992; Barth, 1999; Barth & Schatzmayr, 1992; Gubler & Kuno, 1997; Henchal & Putnak, 1990; Rice 1996).

Electron microscopy has been used as a very efficient tool to study virus particle morphology (Ackermann & Berthiaume, 1995; Doane & Anderson, 1987; Madeley & Field, 1988), as the unique technique for direct visualization of morphological structures. Virus infected tissues can also be analyzed by this technique (Murphy & Nathanson, 1997). Modern techniques of molecular biology have detected several steps of the dengue virus pathway across infected cells (Rice, 1996; Westaway, 1987). Morphological studies using electron microscopy showed regions inside the cells where virus particles replicate until its (Barth, 1992; Ng & Corner, 1989).

In relation to the genus *Pestivirus*, ultrastructural observations suggest that these viruses, like dengue viruses, remain inside cell vesicles and are released from the Golgi complex by exocytosis (Bielefeldt Oltmann & Bloch, 1982; Gray & Nettleton, 1987). Nevertheless, Hepatitis C virus (HCV) evolution inside infected cells was not yet extensively studied by electron microscopy; molecular biology studies of HCV proteins during viral maturation suggest a transit of virus particles through the Golgi system for exocytosis (Rice, 1996); this observation agrees with our model for dengue virus replication (Barth, 1992; Barth, 1999).

However, budding of *Flaviviridae* virus particles into intracellular vesicles, as presented by several authors (Hase *et al.*, 1989; Ng *et al.*,

1994; Rice, 1996) could never be demonstrated in our experiments by electron microscopy. Our data obtained using immunolabelling and *in situ* hybridization experiments with dengue viruses (Grief *et al*., 1997), show that the virus particles are formed inside the lumen of the rough endoplasmic reticulum (rER), with an active participation of the rER-membrane bound ribosomes and vesicles and tubules of smooth-membrane structures (SMS).

In order to better understand the *Flavivirus* lifecycle, we elaborated a model of virus replication (Barth, 1992; Barth, 1999) using a member of this genus, dengue virus type 2 (DEN-2, dengue-2), isolated from human sera and inoculated in the clone C6/36 mosquito cell line from *Aedes albopictus*. These studies were carried out by light (LM), confocal (LSCM), scanning (SEM) and transmission (TEM) electron microscopy. We used classical techniques for electron microscope preparations (Maunsbach & Afzelius, 1999), as well as cytochemical identification of cell structures (Robinson, 1985; Spring & Trendelenburg, 1991), immunological viral protein detection (Barth, 1999), *in situ* hybridization of viral RNA (Grief *et al*., 1997) and ultrathin cryosections.

The lifecycle of the dengue virus is demonstrated by high resolution pictures in the present atlas. Additional informations are presented in the legends of the figures.

For light microscope observations a Zeiss-Axiophot microscope equipped with differential interference contrast and FITC-fluorescence filter was used. A Zeiss-laser-scan-confocal microscope was used for additional fluorescent observations. The scanning electron microscope images were obtained with a Zeiss-DSM 940 instrument, equipped with secondary electron and back-scattered electron detectors. The transmission electron microscope images, apart from a few pictures, were obtained with a Zeiss-EM 900 instrument.

ABREVIATIONS

DIC - differential interference contrast (Nomarski's contrast)
DNA - desoxiribonucleic acid
F - filaments
FL - fluorescence
G - Golgi complex
IEM - immunoelectron microscopy
ISH - in situ hybridisation
L - lysosome
LM - light microscopy
LSCM - laser scanning confocal microscopy
M - mitochondrium
N - nucleus
n - nucleolus
P - phagosome
rER - rough endoplasmic reticulum
RNA - ribonucleic acid
SEM - scanning electron microscopy
SMS - smooth membrane structures
TEM - transmission electron microscopy
V - vacuole

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I would like to acknowledge first my husband, Dr. Hermann G. Schatzmayr, head of the Department of Virology, Instituto Oswaldo Cruz, that encourage me to prepare this book; also to my colleague Mrs. Luzia M. de C. Côrtes for cell and virus cultures processing and also for the ultrathin preparations. I would also like to thank the staff of the Flavivirus Laboratory of our Department, in special to Dr. Rita M. R. Nogueira, for all the facilities provided; to Dr. Christopher Grief for IME and EM-ISH preparations; to Mrs. Maria da P. R. Costa for photographic support; to the staff of the Department of Pathology for LSCM images; to Mr. Guilherme Ashton (CICT/FIOCRUZ) for computer elaborated figures 1 and 88; to Mr. Genilton J. Vieira, Laboratory for Production and Processing of Images of our Institut, for all the facilities for computer processing; to Mrs. Heloisa Diniz of the same Laboratory for a fraternal cooperation by the time of the image processing and for the elaboration of the Atlas coverpage; to Dr. M. R. Miagostovich of the Flavivirus Laboratory, Instituto Oswaldo Cruz, for text revision. I am indebted also to Prof. Dr. Kurt Bienz and Mrs. Denise Egger of the Department of Virology, Institute for Medical Microbiology, University of Basel, Switzerland for helpful discussions on our data. Some of the figures were published formerly in Acta Microscopica (Barth & Côrtes, 1997; Barth *et al*., 1997), Archives of Virology (Grief *et al*., 1997), Memórias do Instituto Oswaldo Cruz (Barth, 1992; Barth & Schatzmayr, 1992) and Journal of Submicroscopic Cytology and Pathology (Barth, 1999) and are reproduced by kind permission of these journals. Financial support was obtained by the Instituto Oswaldo Cruz of the Oswaldo Cruz Foundation (FIOCRUZ) (Director: Prof. Dr. José Rodrigues Coura), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Banco do Brasil Foundation and the Ministry of Health, Brazil.

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FIGURES

The virus particle

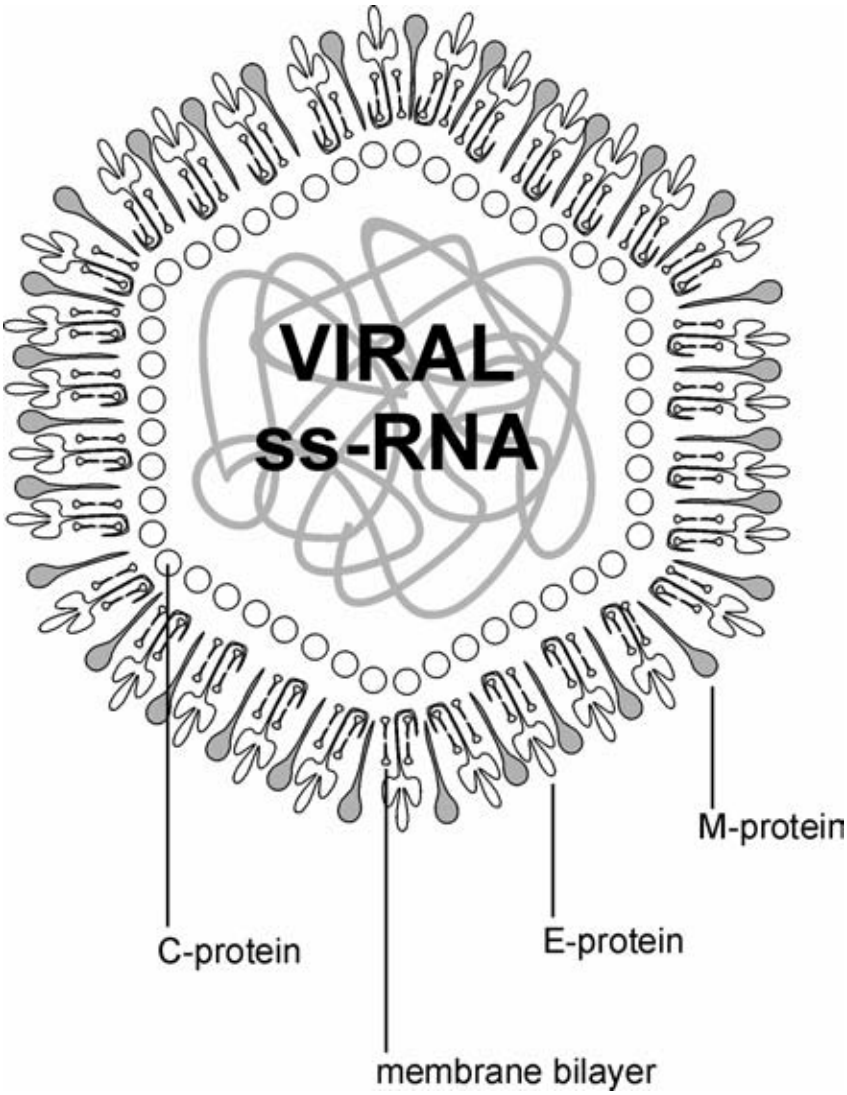
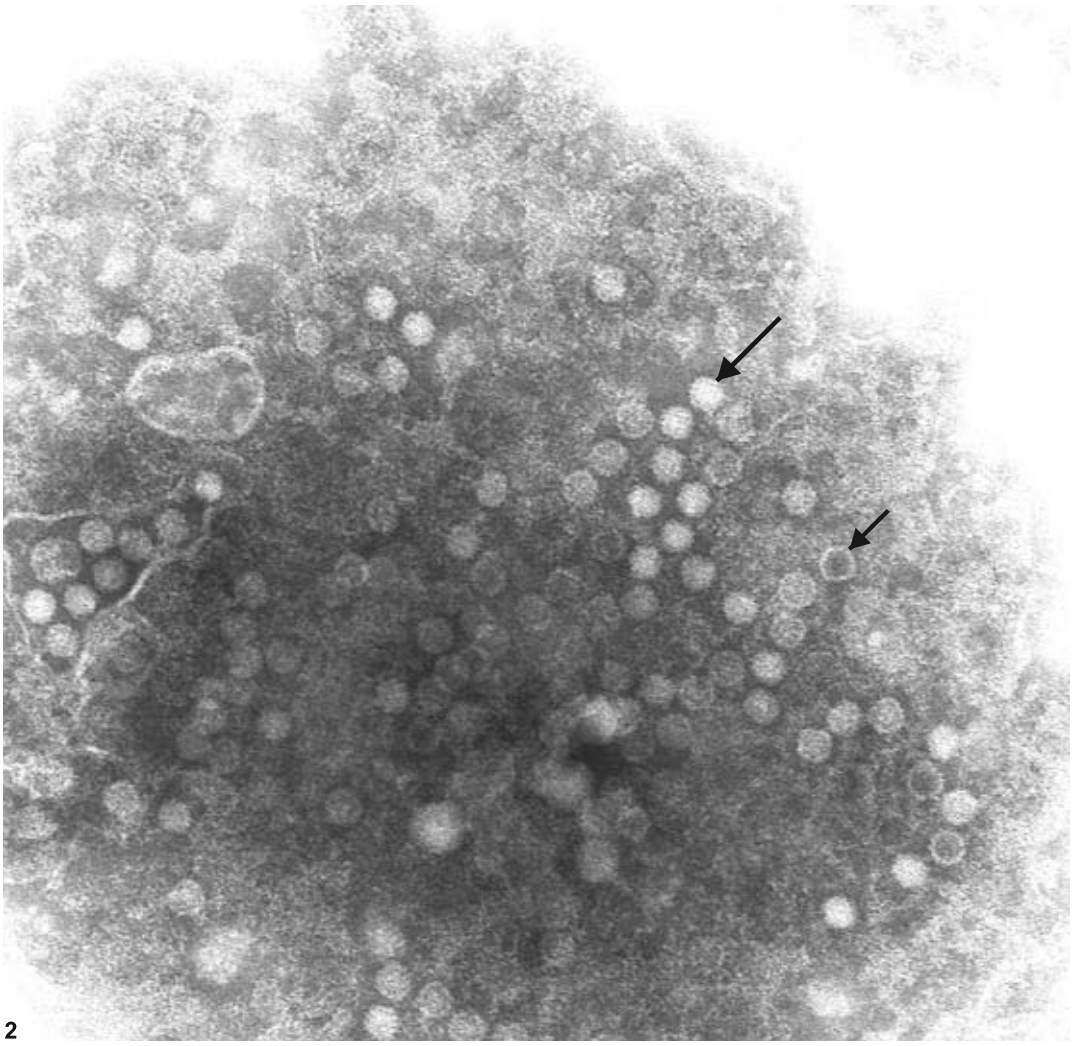


Fig. 1. Model of a dengue virus particle (virion).

The viral genome comprises a positive single-stranded ribonucleic acid chain (ssRNA), surrounded by a core protein (C-protein), forming the viral nucleocapsid. A lipid bi-layer, which contains the structural membrane (M-protein) and envelope (E-protein) proteins, forms the virus particle envelope. After the clivage of the precursor M-protein (prM-protein) during virus particle exocytosis, the M-protein confers infectivity to the virus particle. The viral core diameter is ca. 30nm and the virion diameter ca. 65nm. Magnification: ca. 1,500,000x

The virus particle



2

Fig. 2. Dengue-2 virus infected mosquito cell culture. TEM

The cells were frozen twice at the sixth day post-infecion. The cell/virus suspension was clarified by low speed centrifugation. The resulting fluid was precipitated with amonium sulphate and high speed centrifuged for 15 min. The pellet was resuspended in a drop of destilled water and negatively stained with 2% PTA, pH 7.2 (Barth, 1984, 1985).

Original magnification: 85,000x. Final magnification: 170,000x.

Empty (small arrow) and nucleic acid containing virus particles (large arrow) are assembled together with cytoplasmic debris. One vesicle, at the left side of the figure, contains numerous virus particles of different electron density. Viral envelope proteins are visible as little bright spots on the virus particle envelopes.

The virus particle

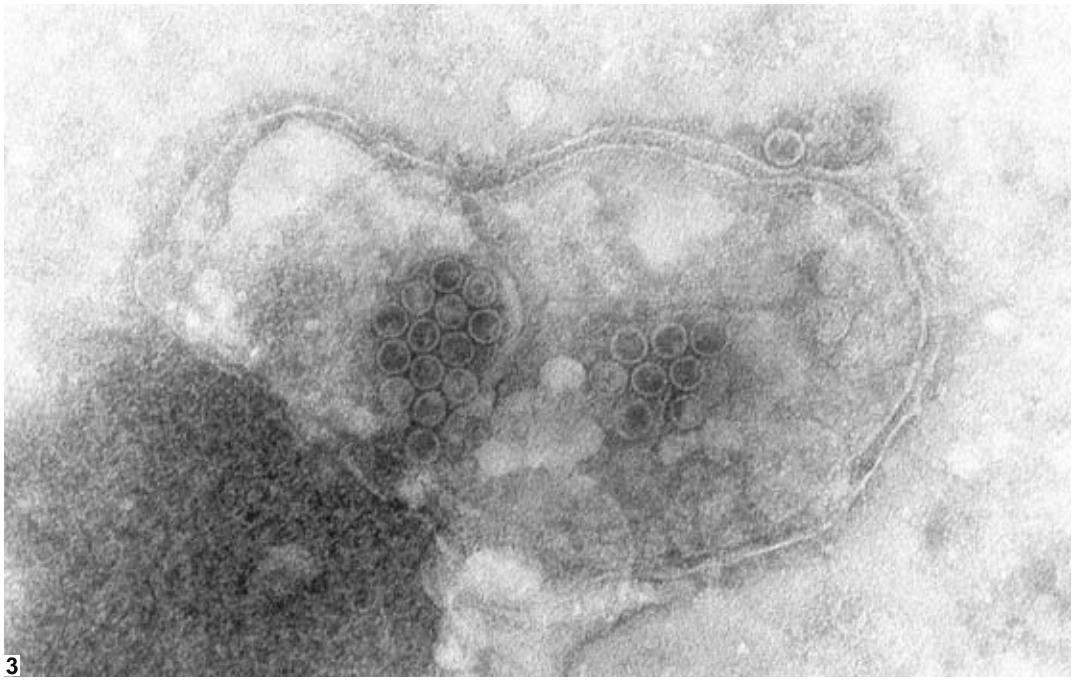


Fig. 3. Dengue-2 virus infected mosquito cell culture. TEM

The infected cell suspension was ultracentrifuged during 1h at 3,000rpm. The pellet was resuspended in destilled water and negatively stained with 2% PTA, pH 7.2.

Original magnification: 52,500x. Final magnification: 140,000x.

Groups of incomplete (“empty” or “partially empty”) virus particles are enclosed in a cytoplasmatic vesicle. One “empty” virus particle and cell debris lay outside this vesicle.

The virus particle

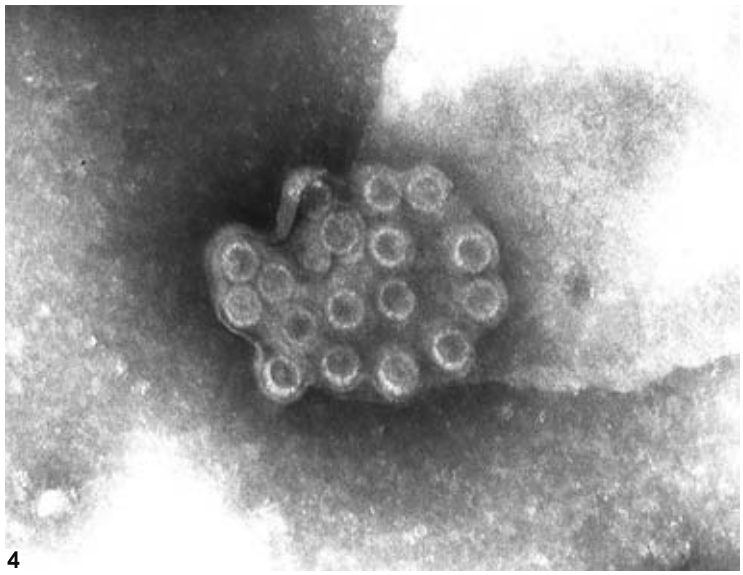


Fig. 4. Dengue-1 virus infected mosquito cell culture. TEM

The same methodology as described in Fig. 3 was used.

Original magnification: 52,500x. Final magnification: 140,000x.

Virus particles inside a cell vesicle. The majority of the virus particles is lacking nucleocapsids and presents the membrane and envelope proteins as bright dots in their envelopes.

The virus particle

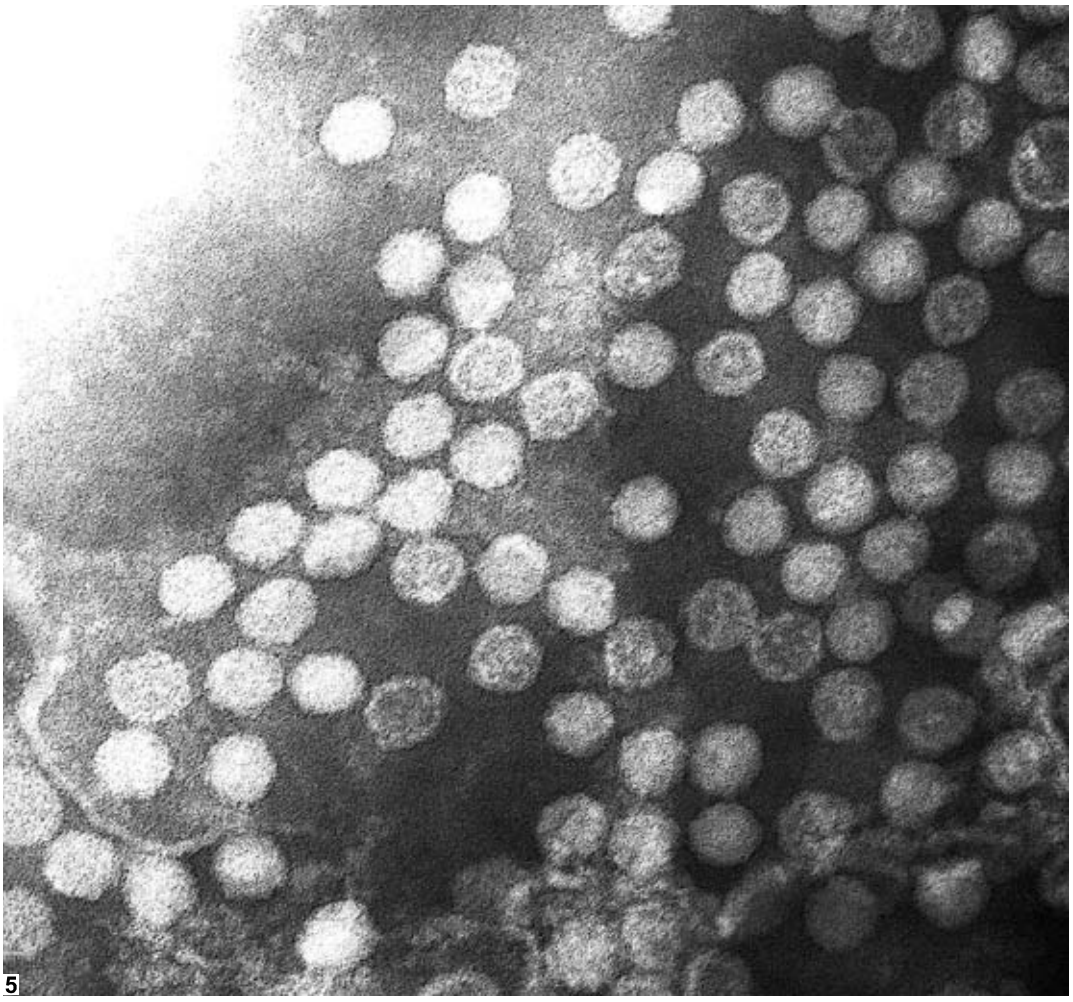


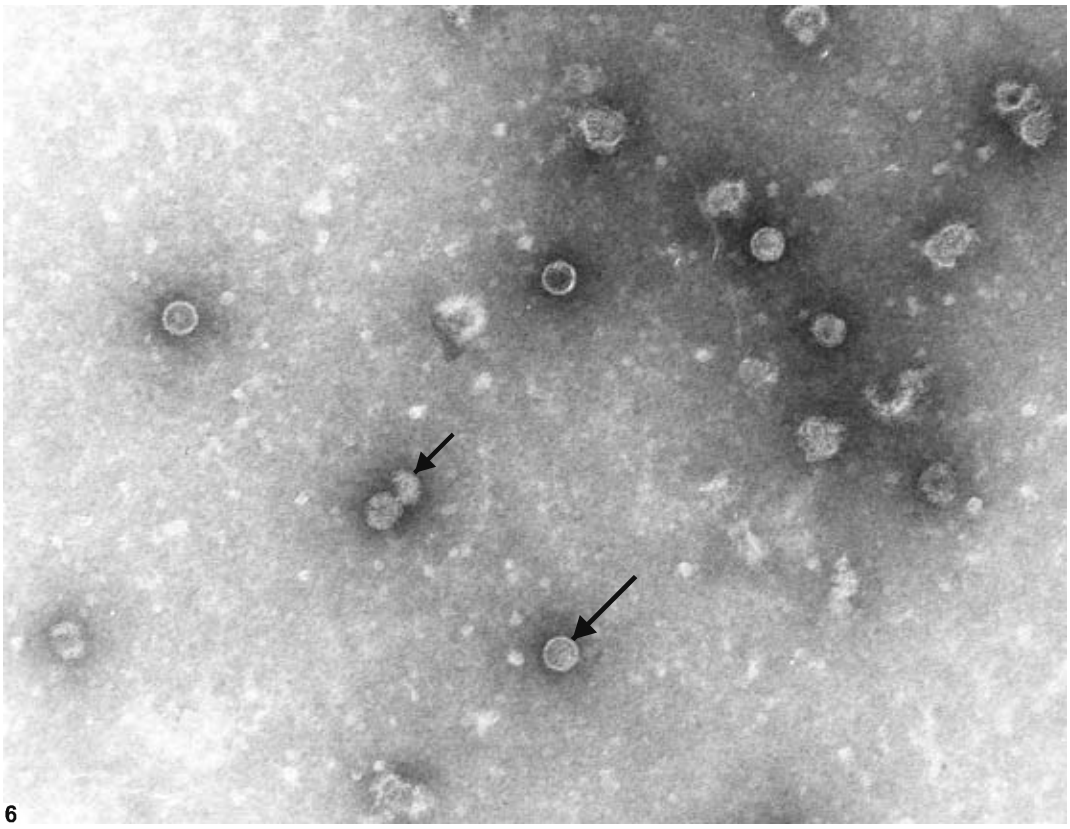
Fig. 5. Dengue-2 virus infected mosquito cell culture. TEM

The same methodology as in Fig. 2 was used.

Original magnification: 140.000x. Final magnification: 340,000x.

Different sized virus particles, with or without cores, are assembled by high speed centrifugation only. The structure E and M-proteins are better emphazised in “empty” virus particles.

The virus particle



6

Fig. 6. Dengue-1 virus infected mosquito cell culture. TEM

The infected cell cultures of ten Roux flasks mixed together were clarified and purified in a saccharose gradient; fraction 21 was negatively stained with 2% PTA, pH 7.2 and illustrated.

Original magnification: 52,500x. Final magnification: 125,000x.

Virus particles (long arrow) and cell membrane debris (small arrow) are mixed. Cell membrane fragments form little vesicles, irregularly sized and fuzzy coated, that are distinct from virus particles, even “empty” ones.

The virus particle

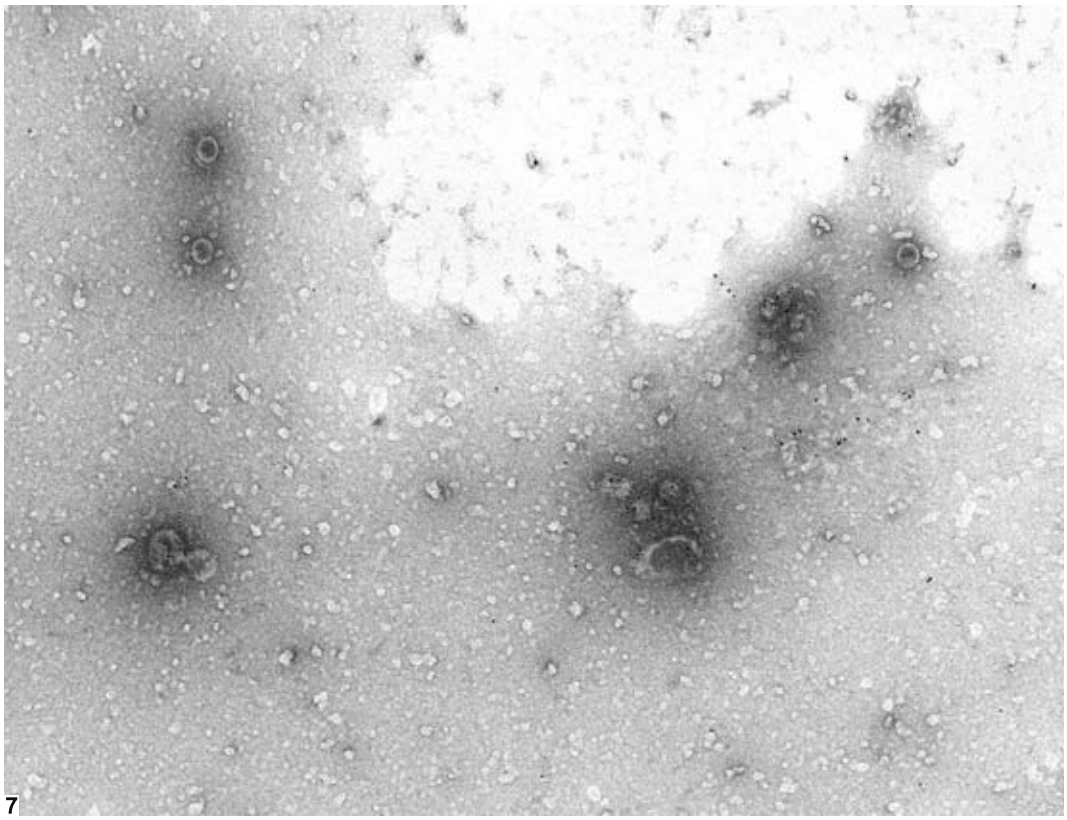


Fig. 7. Dengue-1 virus infected mosquito cell culture. IEM

The same gradient fraction as in Fig. 6. Before negatively stained, the electron microscope grid with the virus particles on, was floated over a drop of a hyperimmune mouse ascitic fluid reactive to dengue viruses, incubated for 30 min at 36°C, followed by incubation with protein A-gold (10 nm) also for 30 min at room temperature.

Original magnification: 30,000x. Final magnification: 75,000x.

Gold particles are linked to the antibody molecules by the protein A. Note that “empty” virus particles are not labelled. Gold linked dengue-1 antibodies, not linked to the virus particles, remain free in the gradient fraction.

The virus particle

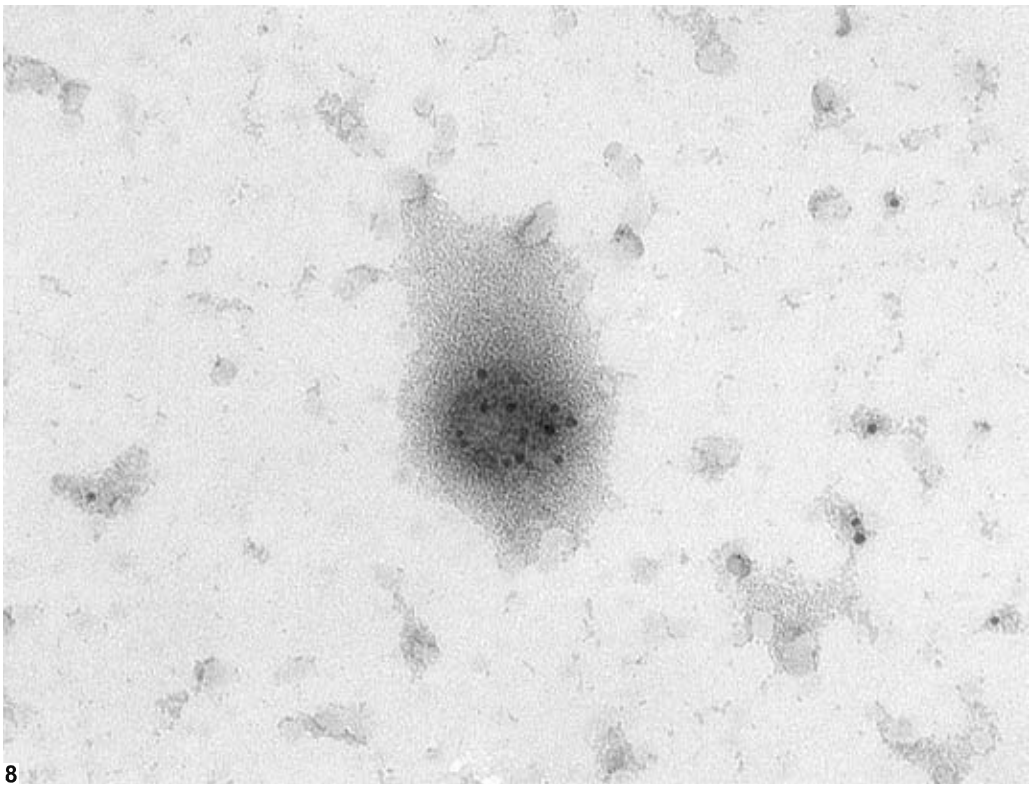


Fig. 8. Dengue-2 virus infected mosquito cell culture. IEM

A saccharose gradient was prepared as described in Fig. 6. The virus particles from fraction 22 were incubated with the specific antibody and protein A-gold as described in Fig. 7. Negative staining was carried out with 2% PTA, pH 7.2.

Original magnification: 85,000x. Final magnification: 230,000x.

Gold particles are linked by antibody molecules to the virus particle surface, which morphological features are no more visible.

The virus particle

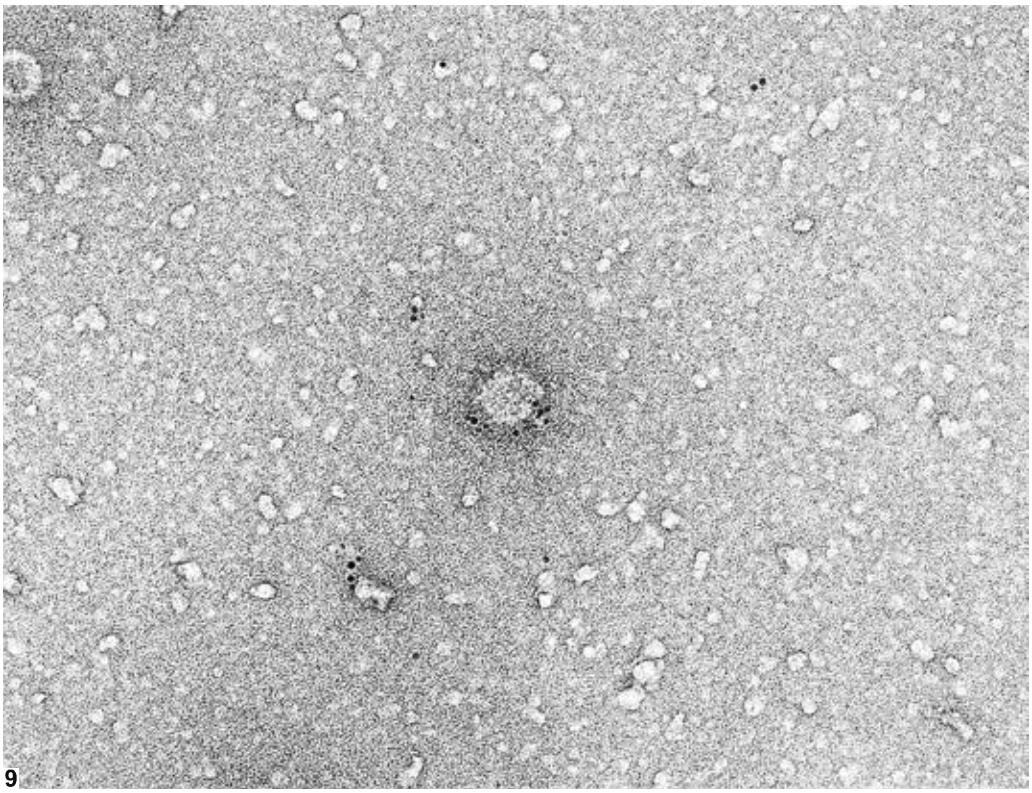


Fig. 9. Dengue-2 virus infected mosquito cell culture. IEM

The same gradient fraction and grid processing as described in Fig. 8. The negative staining was executed with a 2% solution of acqueous uranyl acetate.

Original magnification: 50,000x. Final magnification: 125,000x.

The hard contrast of this figure was effective by the uranyl stain. In both figures (Figs. 8 and 9) some gold-marked antibodies are dispersed all over the grid.

The infected cell

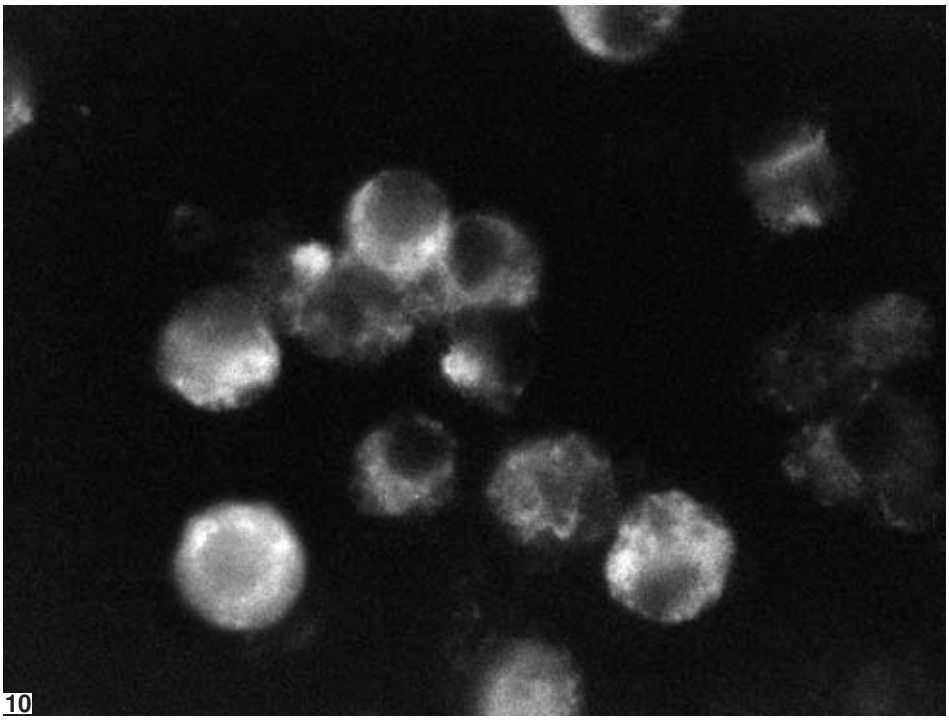


Fig. 10. Dengue-2 virus infected mosquito cell culture. LM/FL

The infected cells were scrapped off from the vial, dropped onto microscope slides, air dried and fixed in cold (-20°C) acetone for 10 min. After incubation (37°C, 30 min) with a dengue-2 specific antibody, immunofluorescence was carried out using a fluorescein conjugated anti-mouse antibody during 30 min at room temperature.

Original magnification: 100x. Final magnification: 400x.

Mostly all the cells are infected by dengue-2 viruses Fluorescence is restricted to the cytoplasm. Sometimes, strong fluorescent dots can be observed next to the cell nucleus, corresponding to cell compartments where strong virus replication is in progress. Controls by omission of the first antibody or of non-infected cell cultures show no fluorescence.

The infected cell

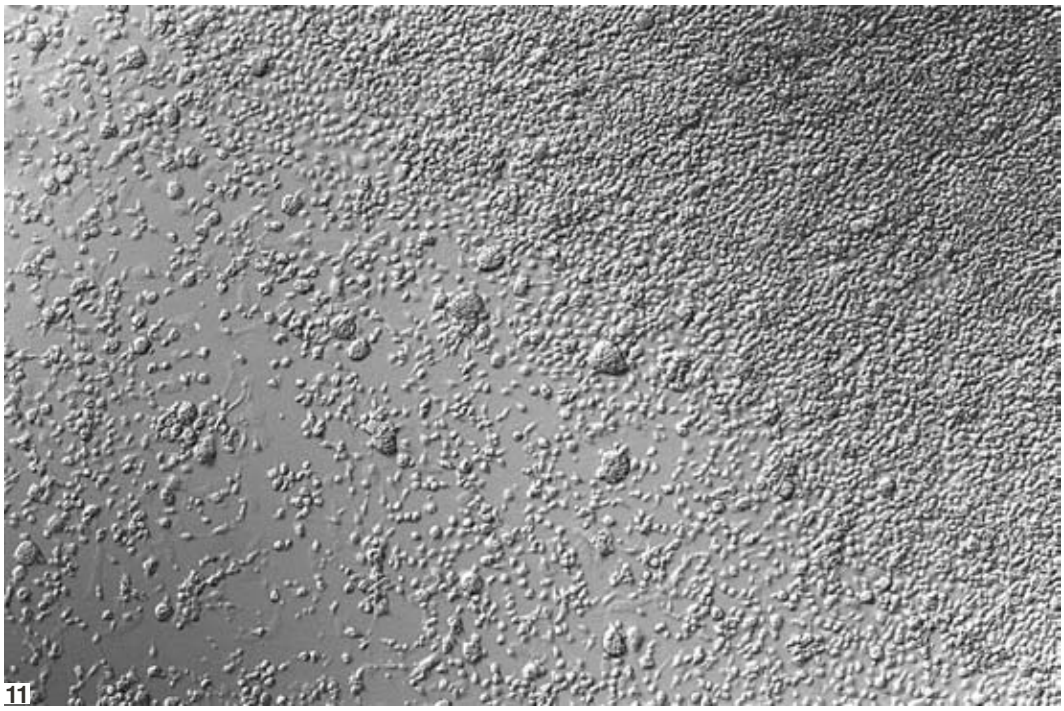


Fig. 11. Dengue-2 virus infected mosquito cell culture. LM/DIC

Infected cell monolayer cultures on cover-glasses were fixed in phosphate-buffered formalin (Millonigs fixative), 72h post-infection, when more or less 30% of cytopathic effect (syncytia formation) could be detected. These samples may be kept for several weeks in the cold (4-8^{o}C) for further processing. After washing in PBS, the samples were photographed in the same buffer.

Original magnification: 50x. Final magnification: 90x.

Virus infection of the cell monolayer was progressing from the lower left to the upper right corner of the figure. Large syncytia are the most important virus factories, visible in the middle part of this figure.

The infected cell

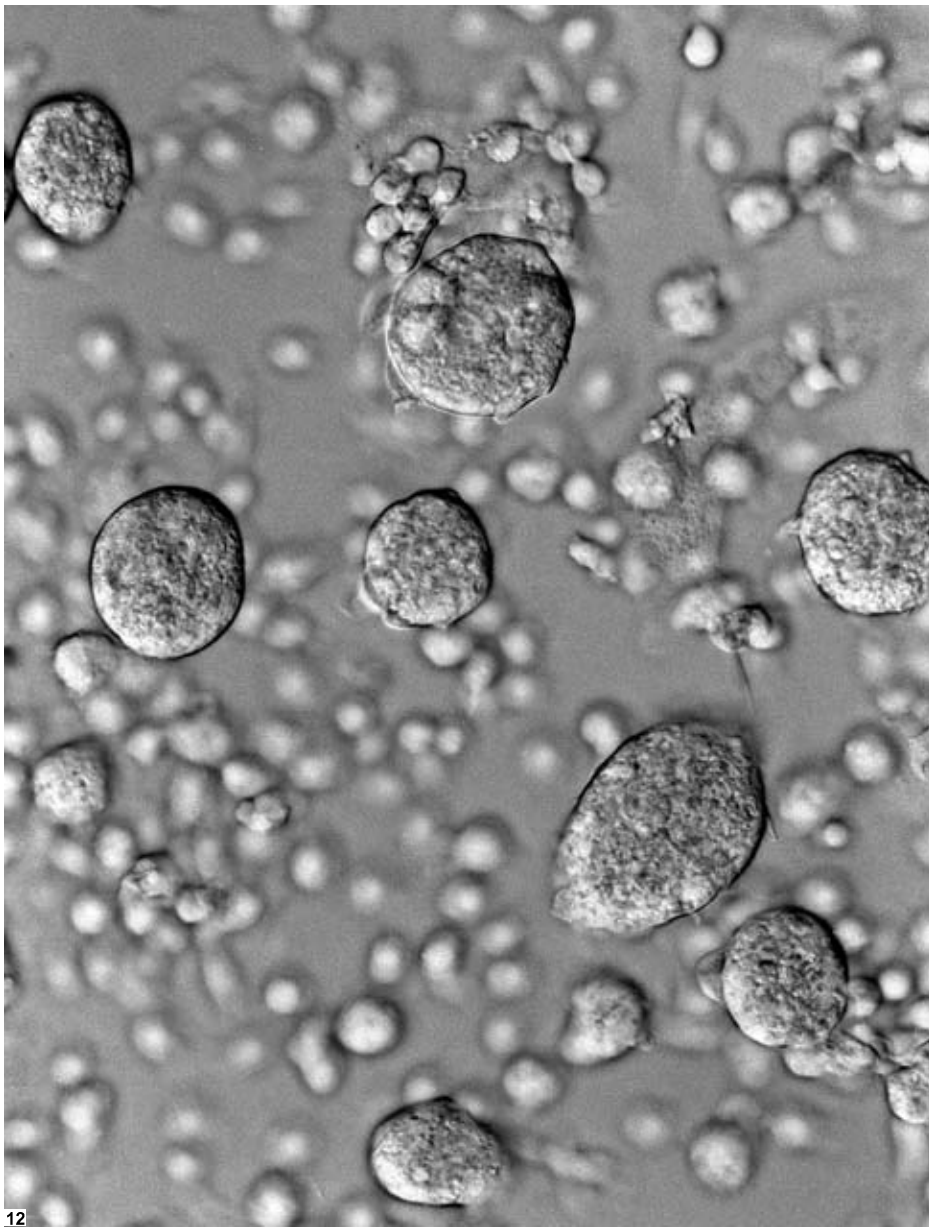


Fig. 12. Dengue-2 virus infected mosquito cell culture. LM/DIC

The same preparation as in Fig. 11.
Original magnification: 100x. Final magnification: 600x.

Fusion of several cells in the presence of virus particles (fusion “from within”) originate rounded, different sized syncytia. Its average diameters are always larger than 25µm, while single non-infected cells have an average diameter of 12,5µm and a maximal diameter of 20µm.

The infected cell

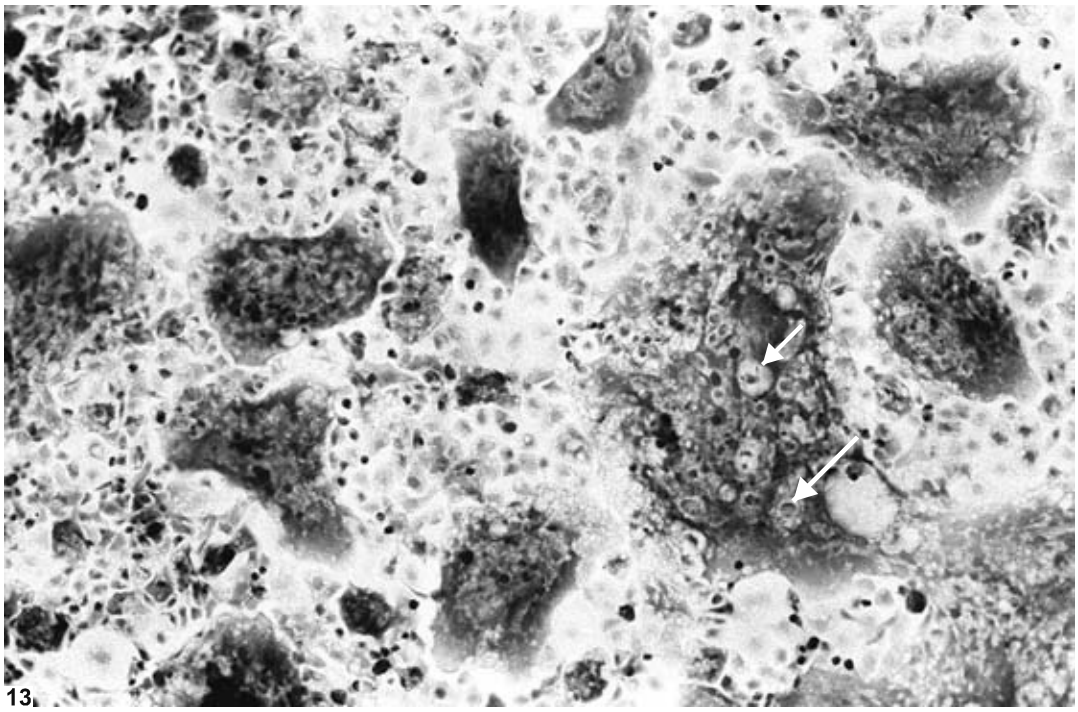


Fig. 13. Dengue-2 virus infected mosquito cell culture. LM

The infected cell monolayer was processed as described in Fig. 11. After removing the fixative, the cells were Giemsa stained and sealed with Damar glue.

Original magnification: 100x. Final magnification: 350x.

This older than in Fig. 11 infected cell culture present larger and irregular shaped syncytia. Cell organelles as nuclei (small arrow) and vacuoles (large arrow) may be distinguished. Non-infected cells occur between the syncytia and may develop to a persistent cell culture infection.

The infected cell

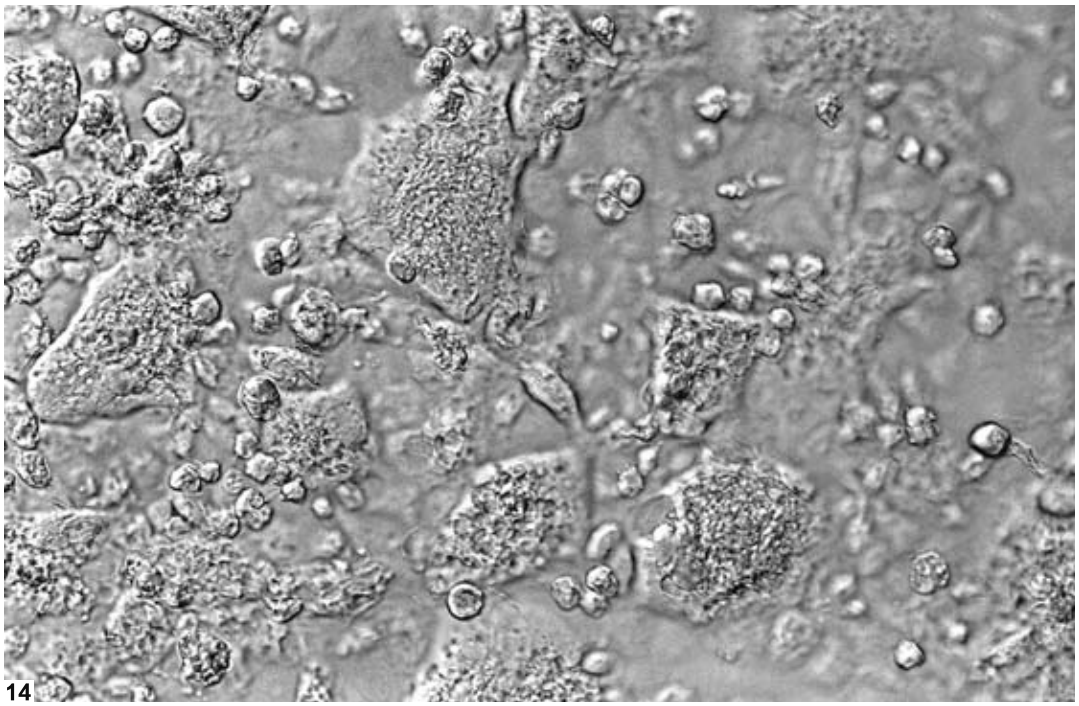


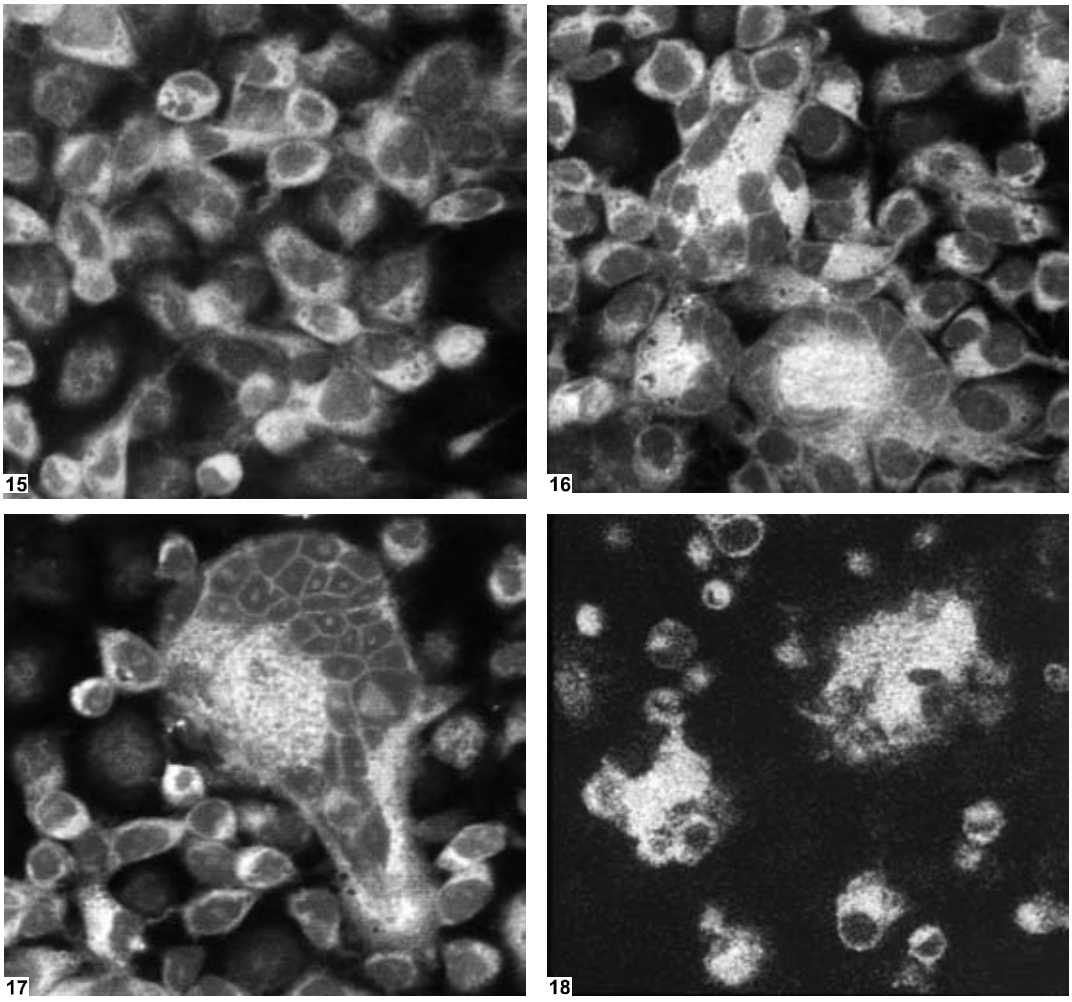
Fig. 14. Dengue-2 virus infected mosquito cell culture. LM/DIC

The same cell culture as in Fig. 13, embedded in phosphate buffer solution, without staining.

Original magnification: 100x. Final magnification: 350x.

The rough surface of the irregularly shaped large syncytia is emphasized.

The infected cell



Figs. 15-18. Dengue-2 virus infected mosquito cell culture. LSCM

Infected cell monolayers were grown on cover-glasses, fixed in Millonigs fixative three days post-infection and processed for laser-scan-confocal microspcopy (LSCM) observation. Cell compartments and organelles may be emphasized using different fluorescent stains. Virus factories are characterized presenting strong antibody and lipid fluorescence, increase of the rough endoplasmic reticulum fluorescence and no or soft RNA fluorescence when compared with the strong fluorescence of cell nuclei.

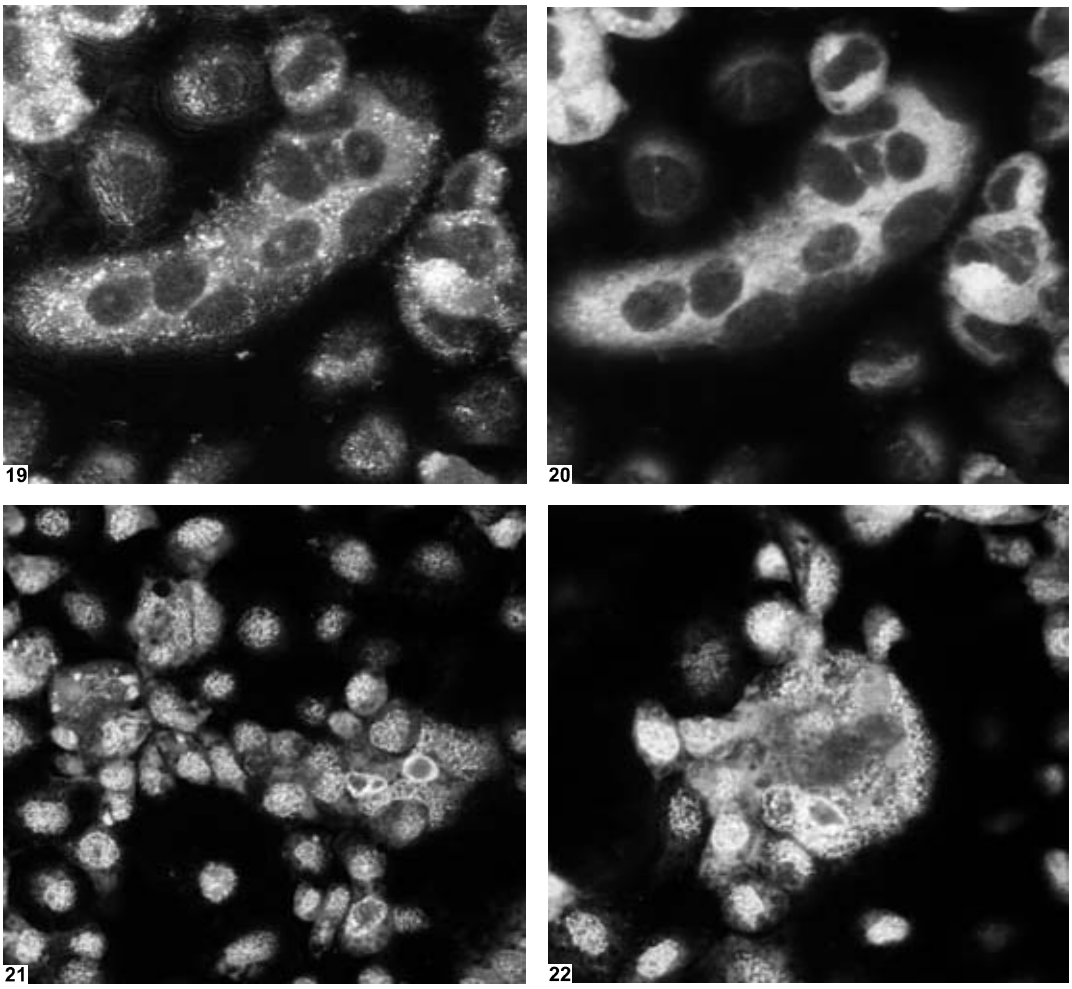
Fig. 15. Nile red staining. Single cells show lipid fluorescence inside the cytoplasm. 300x.

Fig. 16. Nile red staining. Large fluorescent patches inside the syncytia are regions of virus particle factories. 300x.

Fig.17. Nile red staining. A very large syncytium present at the left side the fluorescent region of virus replication and morphogenesis, where lipids are highly concentrated. 300x.

Fig. 18. Fluorescein isothiocyanate (FITC) staining. The cell culture was processed as described in Fig. 10. Antibody fluorescence is restricted to the cytoplasm of single cells and syncytia. 250x.

The infected cell



Figs. 19-22. Dengue-2 virus infected mosquito cell culture. LSCM

Fig. 19. DIOC (3,3-dihexyloxacarbocyanine iodide) staining. This fluorescent stain emphasizes the endoplasmic reticulum in single cells and syncytia. 400x.

Fig. 20. Nile red staining. The same syncytia as in Fig. 19 (double stained), emphasizing high lipid concentration inside the cytoplasm. 400x.

Fig. 21. Acridin orange staining for selective DNA and RNA fluorescence in single cells and syncytia. The fluorescence of nuclei is stronger than of the cell cytoplasm. 200x.

Fig. 22. Acridin orange staining. The cell nuclei are intensively stained. The syncytium presents a softer fluorescent patch in the middle region, where virus morphogenesis is in progress. 250x.

The infected cell

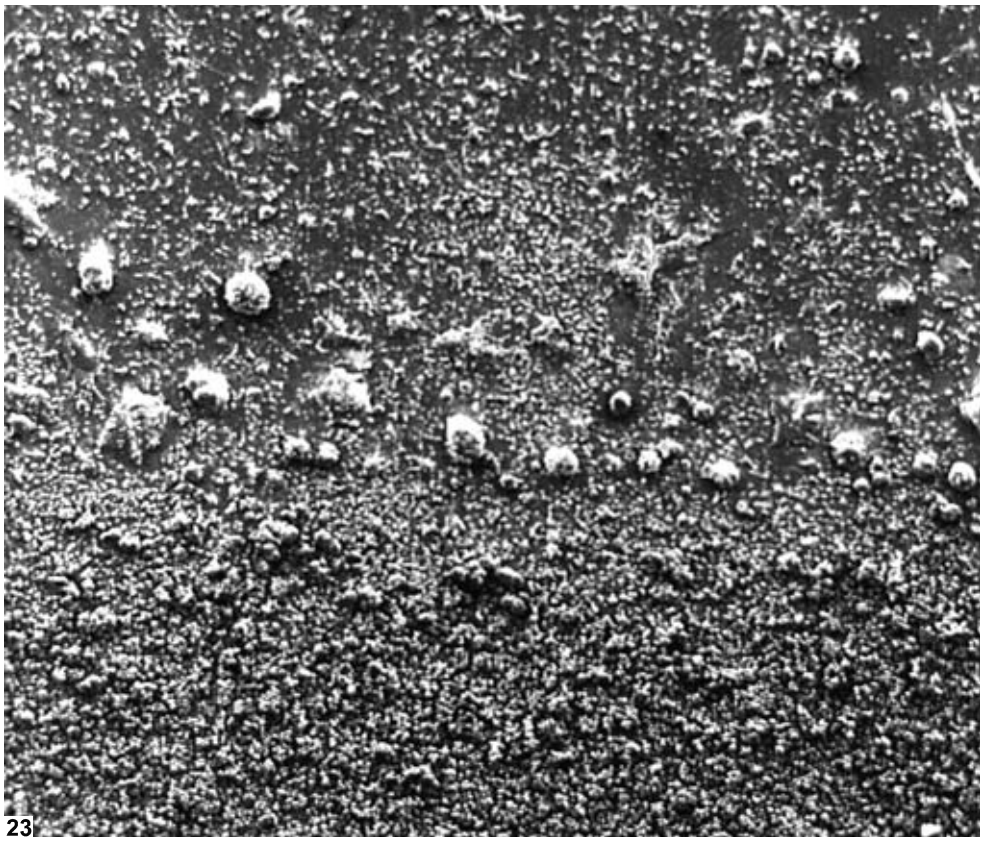


Fig. 23. Dengue-2 virus infected mosquito cell culture. SEM

Infected cell monolayer cultures grown on cover-glasses were fixed in 1% phosphate-buffered glutaraldehyde six days post-infection, when circa 30% of the cytopathic effect (syncytia formation) could be detected. After dehydration with ethanol, the cell cultures were critical point dried and sputtered with a 20 nm thick gold layer. Pictures were taken using secondary electron emission.

Original magnification: 50x. Final magnification: 100x.

Syncytia are frequently of irregular distribution in cell cultures. They appear here as a band in the central part of the figure, while the lower and higher parts of the figure show a dense cell layer, apparently non-affected by virus infection.

The infected cell

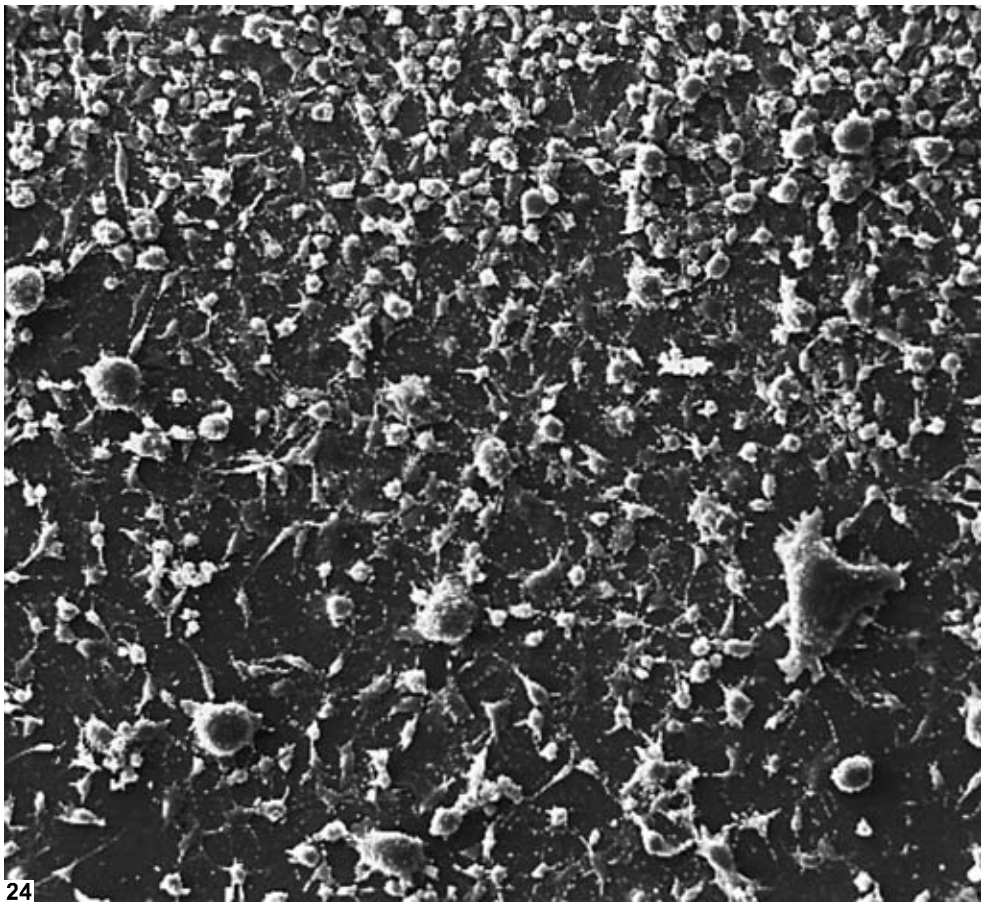


Fig. 24. Dengue-2 virus infected mosquito cell culture.SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 200x. Final magnification: 400x.

Larger and smaller syncytia may be distinguished while cell fusion is progressing. Single, apparently non-infected cells are attached to the cover-glass by long phyllopodia, while syncytia show fewer and shorter cell membrane projections.

The infected cell

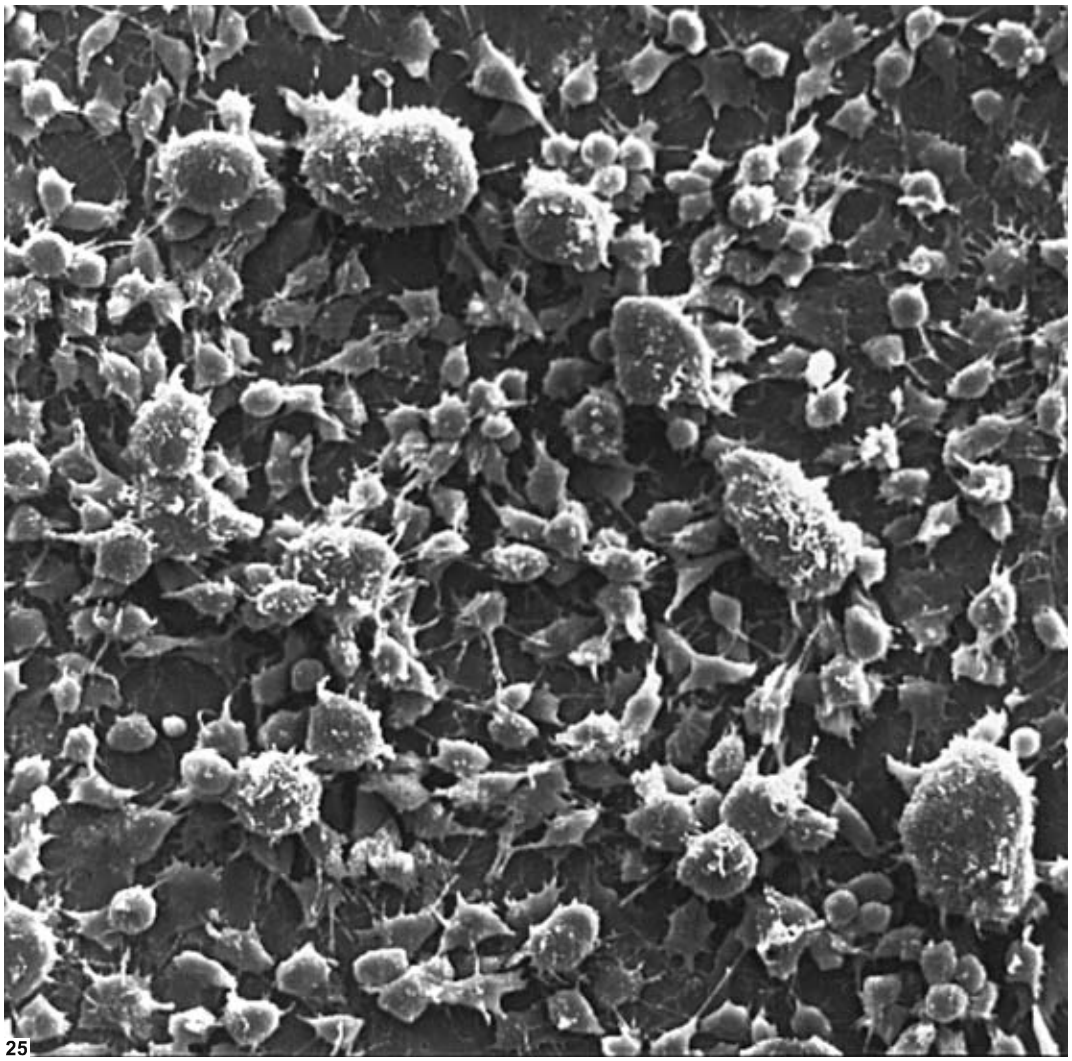


Fig. 25. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 500x. Final magnification: 1,300x.

Fuzzy coated syncytia present a variety of sizes and forms. Long phyllopodia are restricted to single, apparently non-infected small cells.

The infected cell

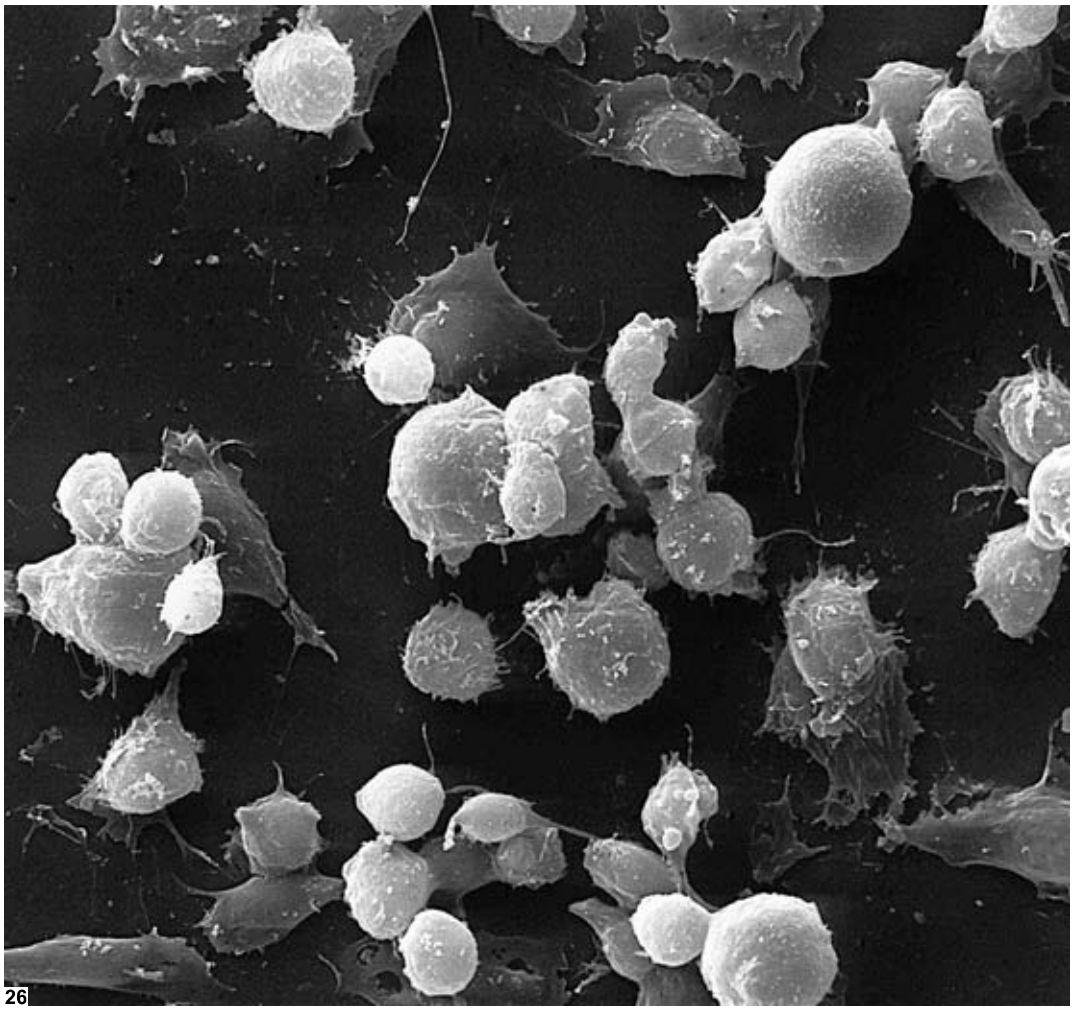


Fig. 26. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 1,000x. Final magnification: 2,600x.

This picture shows a non-confluent cell monolayer. The large syncytium in the upper right is fusioning with two attached cells and shows a scabrous surface; a large vacuole open up and phyllopodia are absent. In the middle part of the figure some cells present numerous phyllopodia and may be not yet infected by the virus. Some cells are spread over the cover-glass surface and present short phyllopodia.

The infected cell

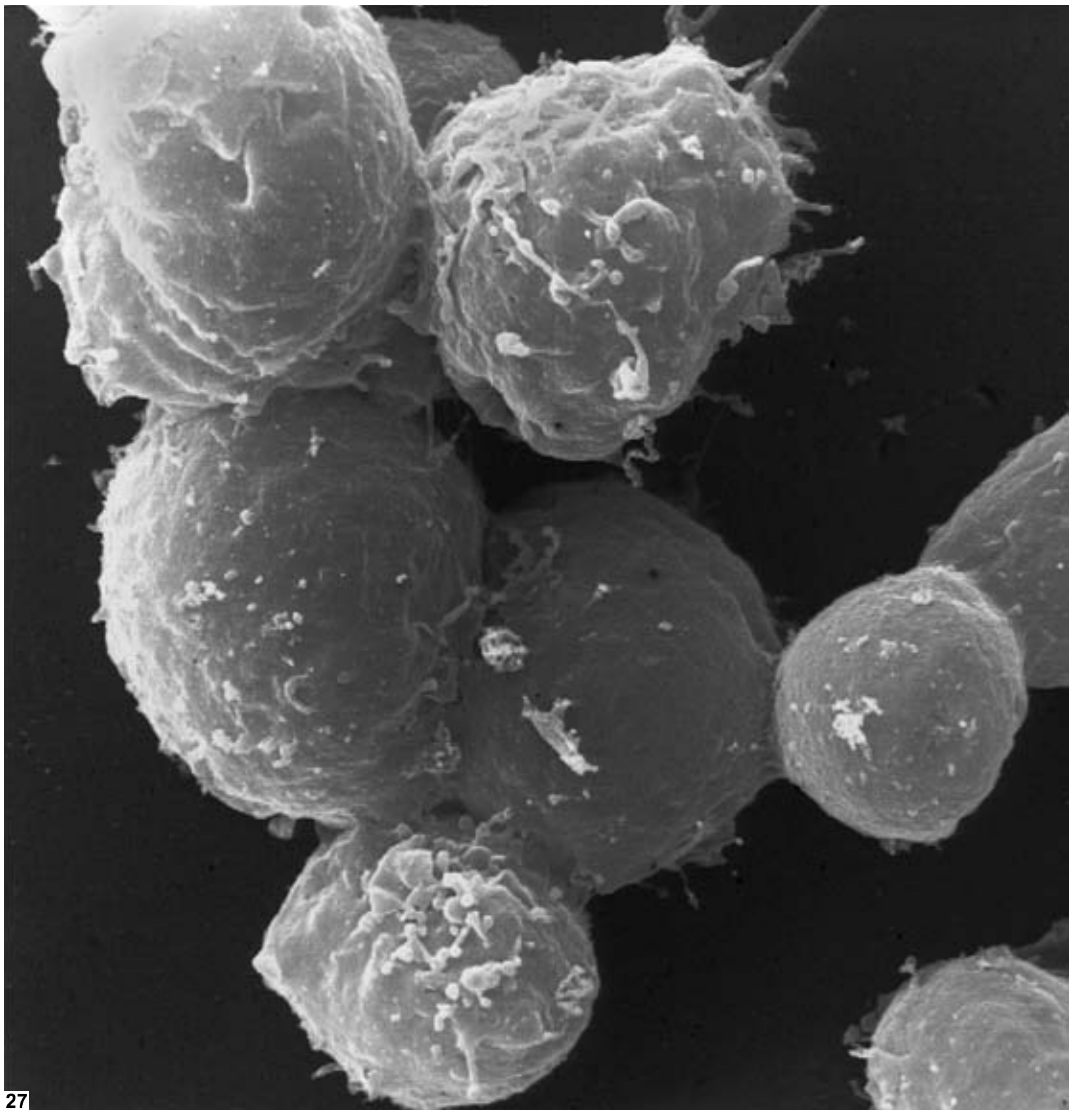


Fig. 27. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 3,500x. Final magnification: 10,500x.

Cell fusion is starting and syncytia magnitude is increased by successive incorporation of single cells. Phyllopodia are yet present on several cells.

The infected cell

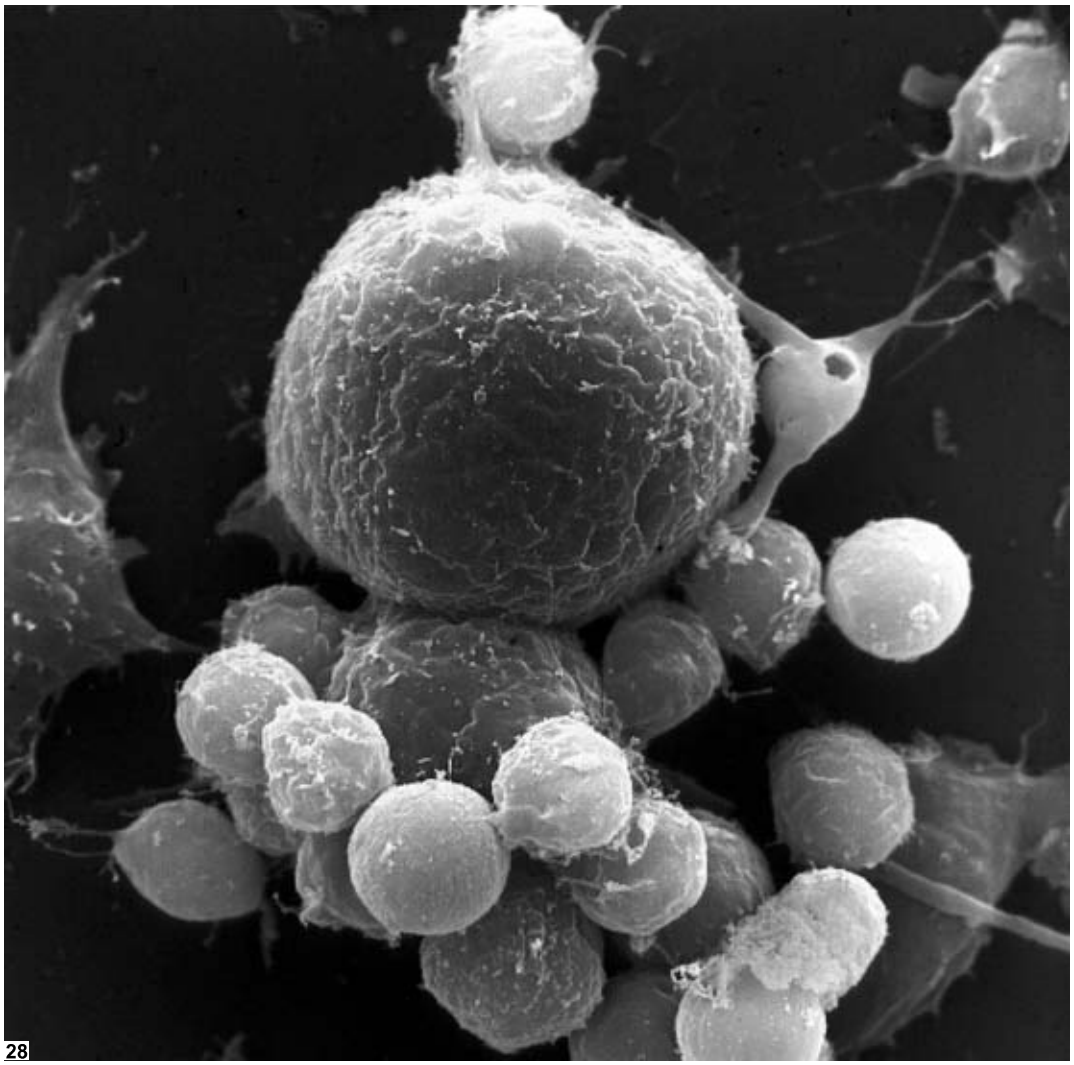


Fig. 28.Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23. A 1/100 diluted antibody was added to the virus containing inoculum. The infected cell culture was fixed 4 days post-infection.

Original magnification: 2,000x. Final magnification: 5,500x.

A large syncytium is fusing with a smaller one. More cells intend to fuse with the syncytia. Small single cells are assembled due to antibody attachment. Phyllopodia are nearly absent.

The infected cell

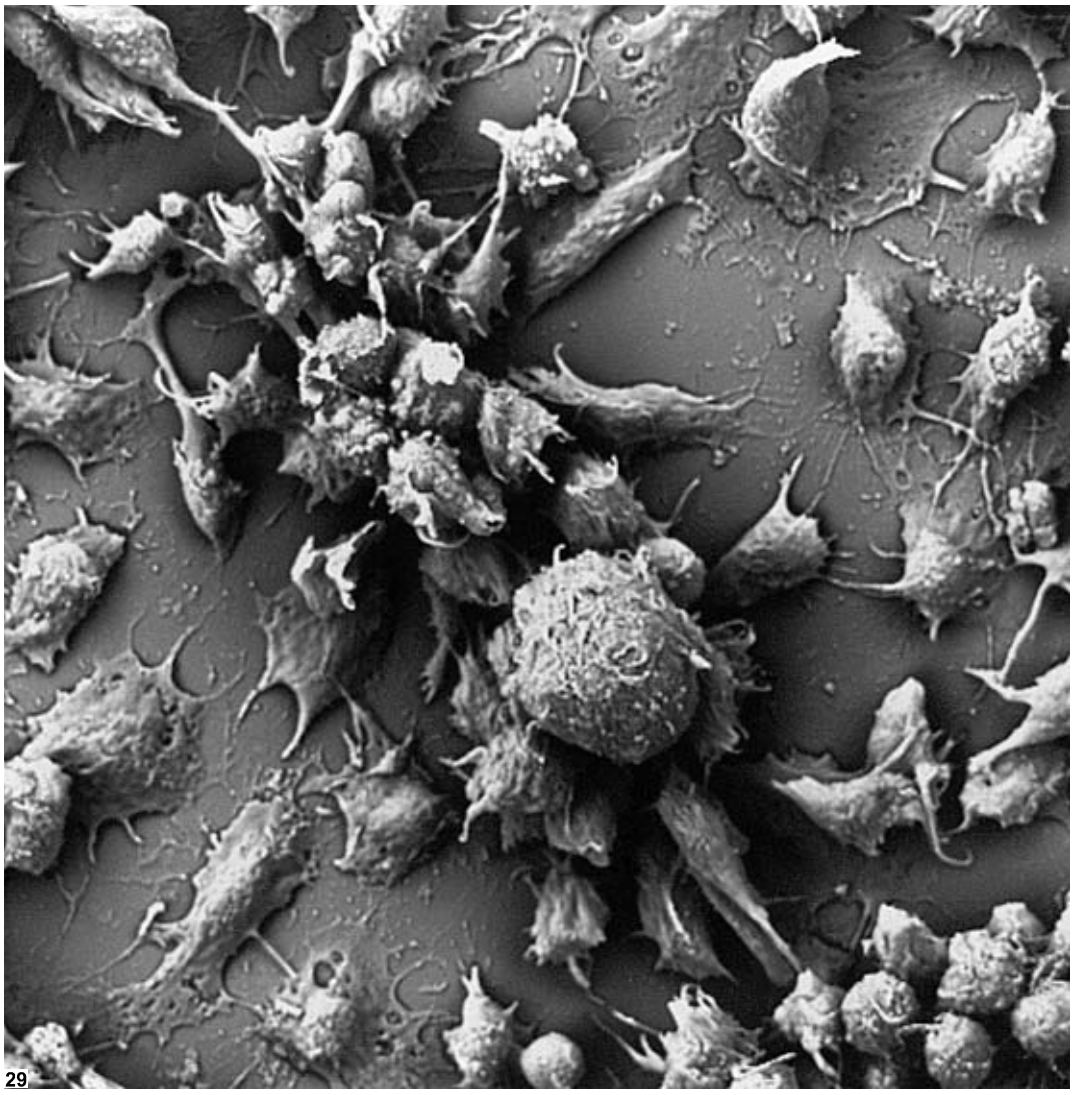


Fig. 29. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 48h post-infection. This image was taken using back-scattered electron emission.

Original magnification: 1,000x. Final magnification: 2,800x.

Syncytia formation starts 24h post-infection. This picture shows two confluent cell groups, one with a centrally located syncytium and the other located above is starting cell fusion. Cells are attached with long phyllopodia to the cover-glass surface.

The infected cell

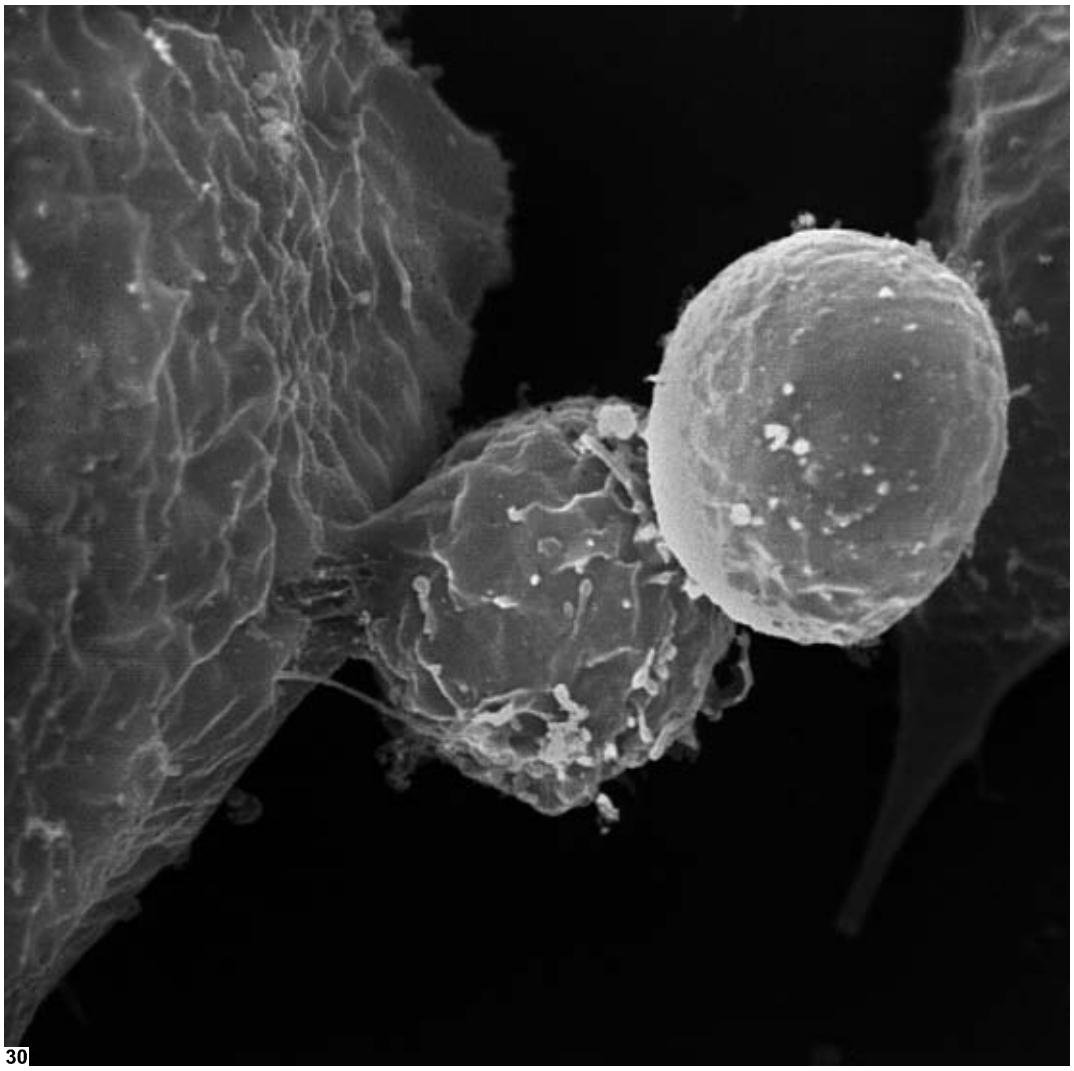


Fig. 30.Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 4 days post-infection.

Original magnification: 5,000x. Final magnification: 14,000x.

A small cell is fusing with a syncytium. Thin phyllopodia are emitted by the small cell and attach to the large syncytium (fusion “from within”; it means the presence of virus particles between the cells). The isolated bright cell shows no more phyllopodia and may be infected also. Compare this fusion aspect with a corresponding TEM image in Fig. 58.

The infected cell

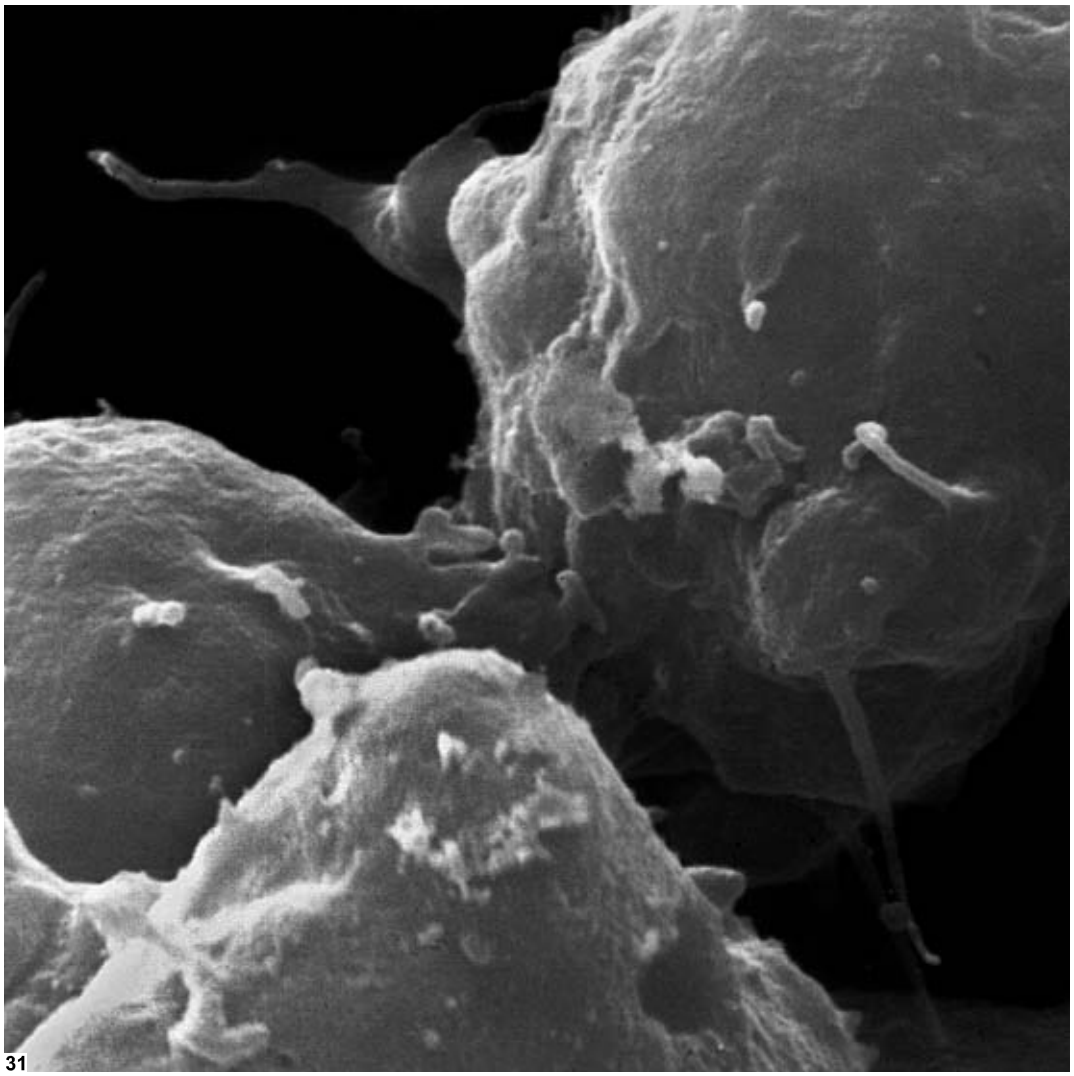


Fig. 31. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 10,000x. Final magnification: 27,000x.

Fusion of two cells is starting by fusion “from within”. The cell at the left directs the phyllopodia of distended hammer-shaped extremities towards the cell at the right. Another cell below shows an open vacuole.

The infected cell

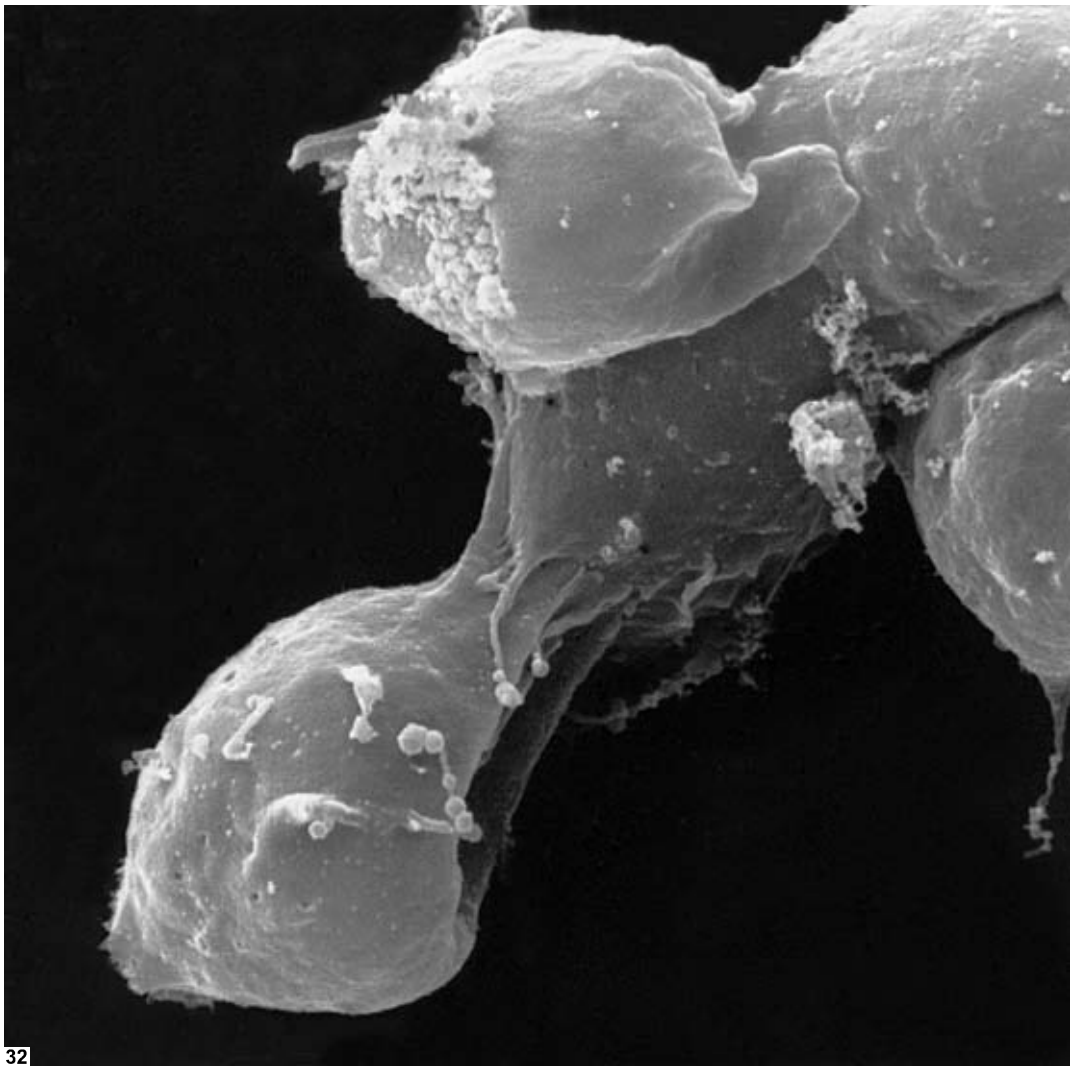


Fig. 32. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 5,000x. Final magnification: 14,000x.

Cell fusion is progressing. Some segments of adjacent cell membranes are fused and few phyllopodia are still distinct. The cell above is broken and shows cell debris at the left side.

The infected cell

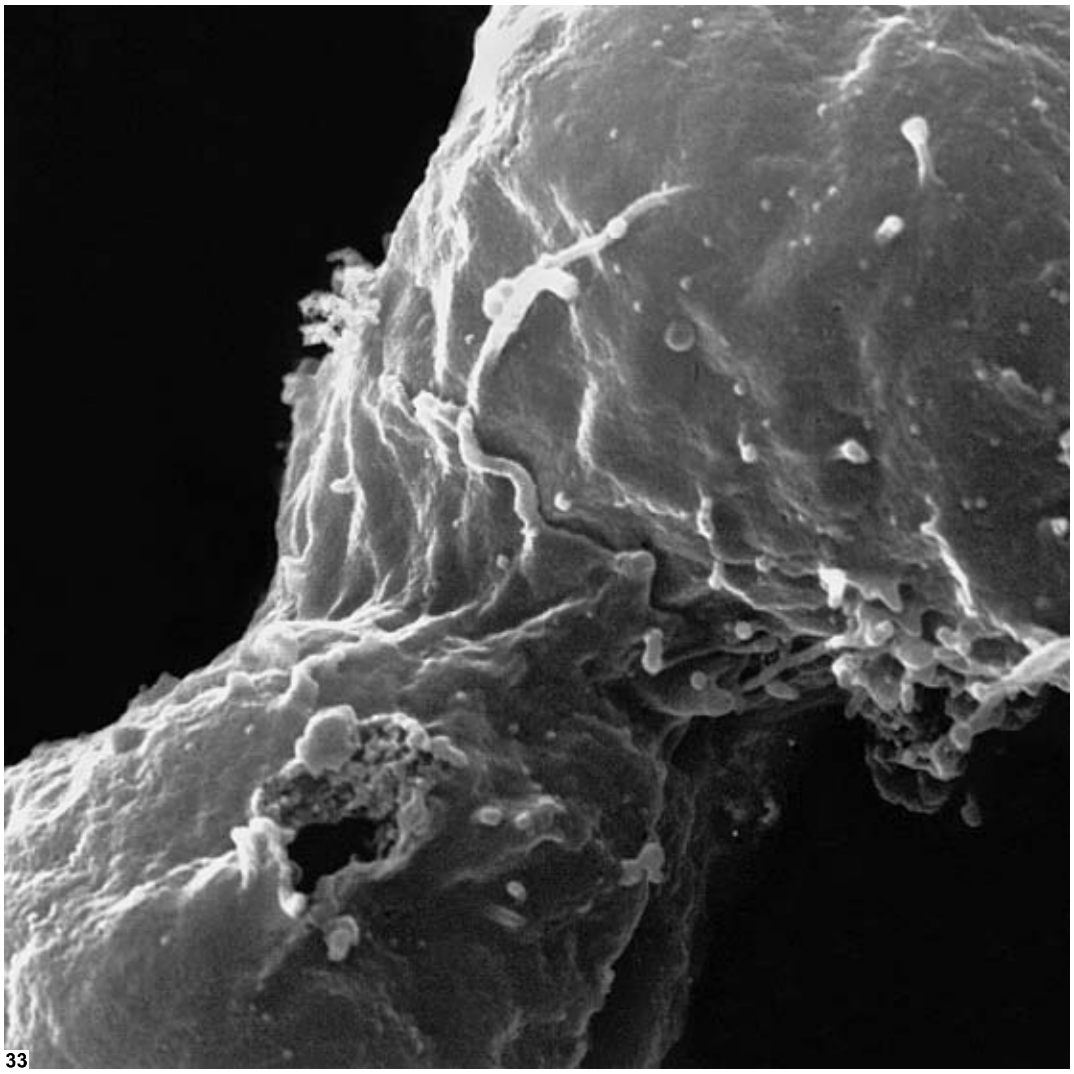


Fig. 33. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 10,000x. Final magnification: 27,000x.

Advanced stage of cell fusion. Few remaining phyllopodia are distinct. The cell below shows an open vacuole.

The infected cell

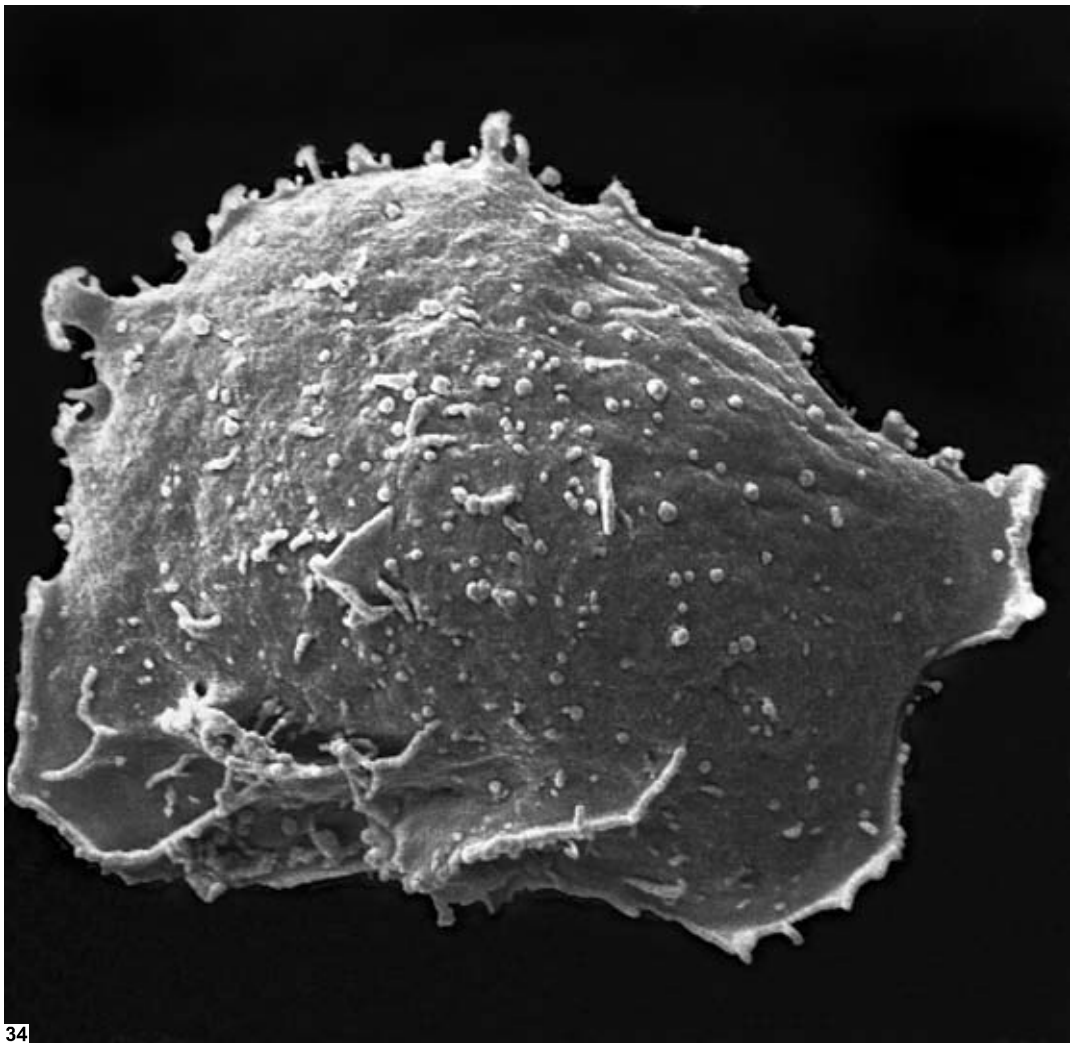


Fig. 34. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 7 days post-infection.

Original magnification: 2,300x. Final magnification: 6,000x.

This large syncytium, presenting short phyllopodia, is flatted and attached to the cover-glass surface. Mixed secondary and back-scattered electron emission were used.

The infected cell

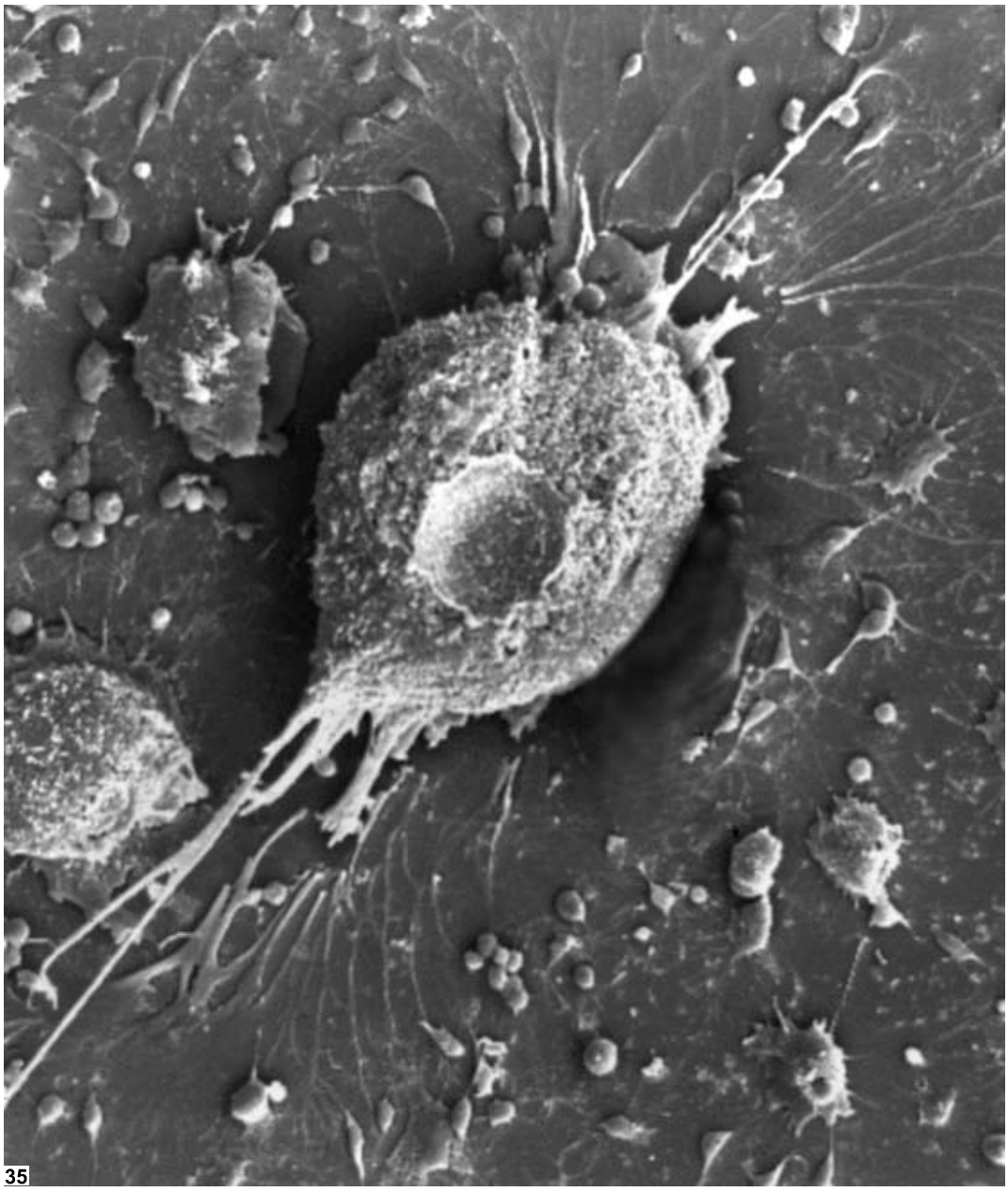


Fig. 35. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 6 days post-infection.

Original magnification: 300x. Final magnification: 900x.

A syncytium is attached with long phyllopodia to the cover-glass surface; a large vacuole is open. The cell monolayer is entirely disrupted at this time of infection, remaining only few single cells and disrupted large syncytia. Mixed secondary and back-scattered electron emission were used.

The infected cell

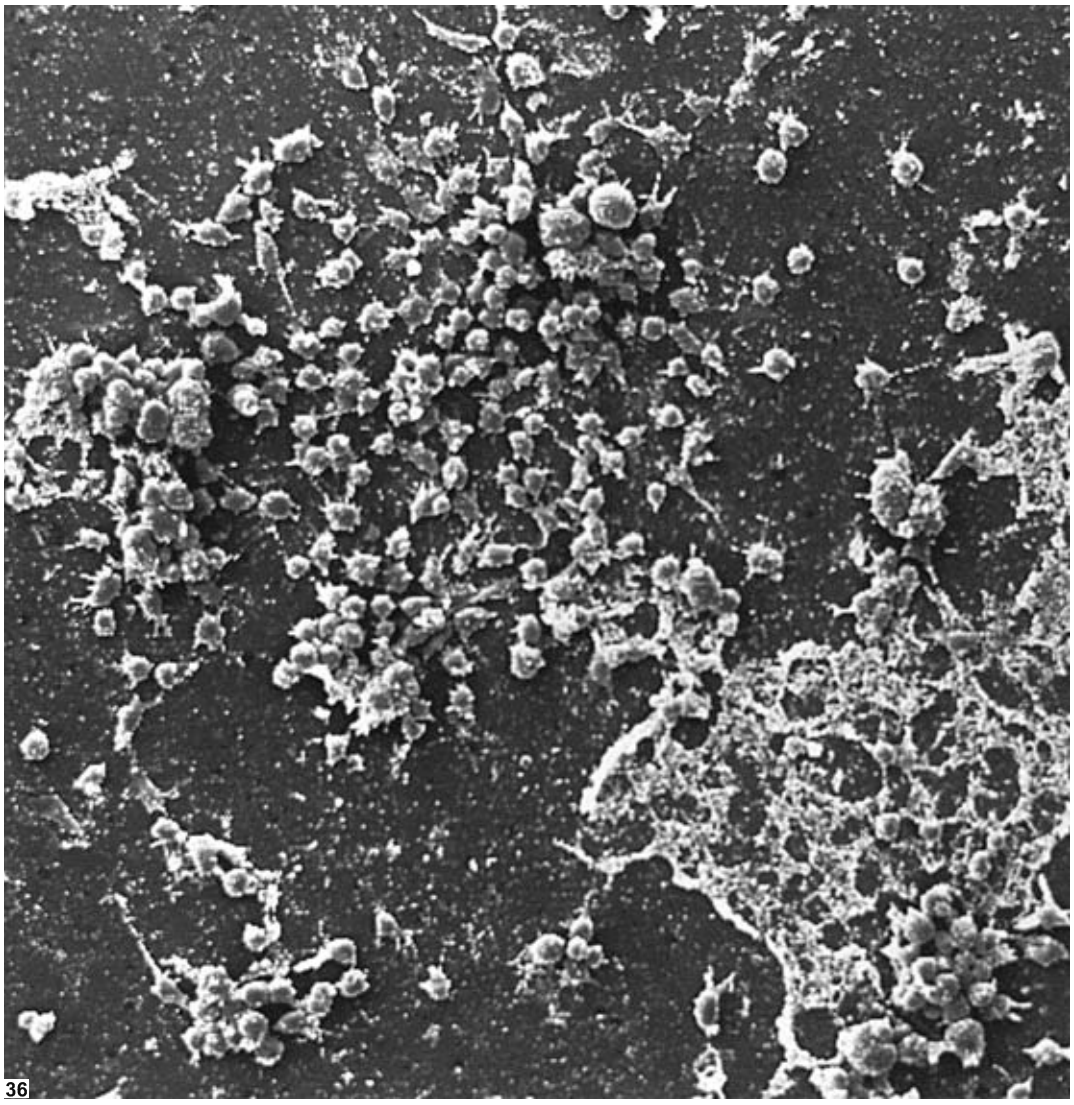


Fig. 36. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 9 days post-infection.

Original magnification: 300x. Final magnification: 900x.

Debris from a large syncytium at the lower right attached to the cover-glass surface. Single cells, not fused, are disorderly grouped and not destroyed cells are also observed, which might progress to a persistently infected cell culture.

The infected cell

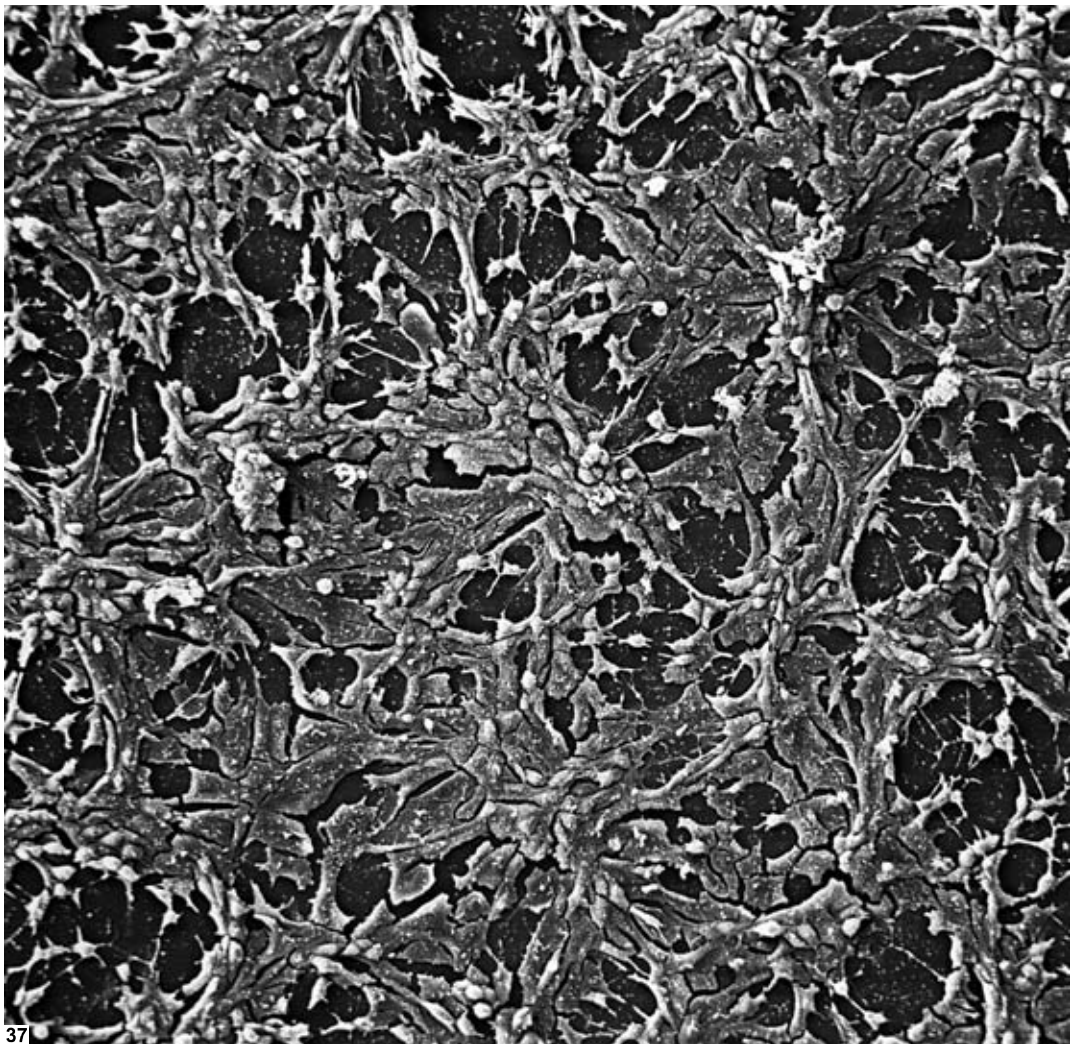


Fig. 37. Dengue-2 virus infected Vero cell culture. SEM

This infected cell monolayer was processed as described in Fig. 23, fixed 72h post-infection.

Original magnification: 100x. Final magnification: 300x.

The confluent monolayer was disrupted by virus infection and some cells are detached from the cover-glass surface. The Vero cell culture does not establish syncytia, nevertheless there are several patches of cell confluence and/or assembling.

The infected cell

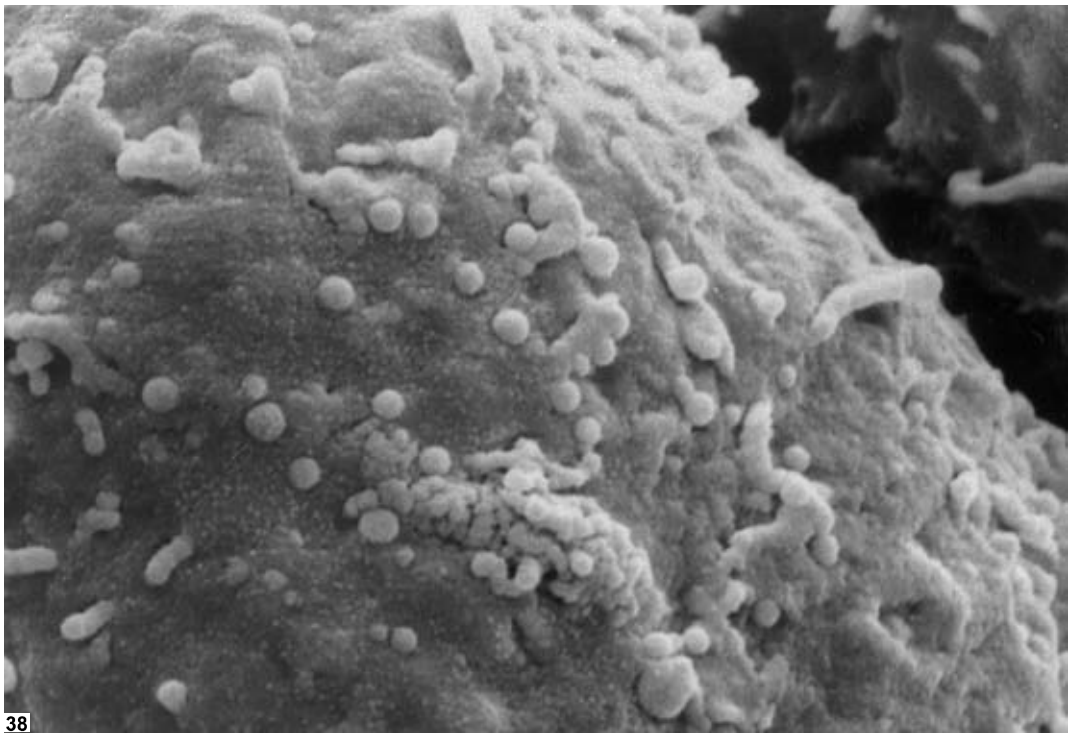


Fig. 38. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 10,000x. Final magnification: 16,000x.

Surface of a large syncytium. The phyllopodia length is reduced. The cell surface has a scabrous appearance. Secondary electron emission of a Zeiss-DSM 942 scanning electron microscope was used.

The infected cell

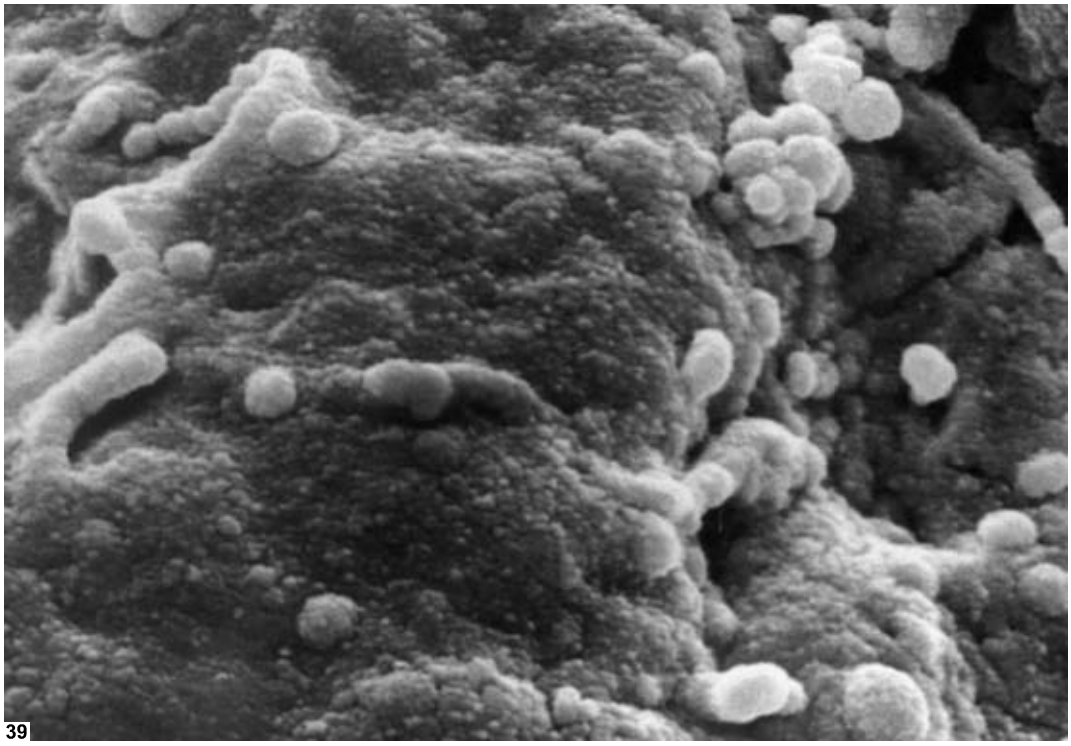


Fig. 39. Dengue-2 virus infected mosquito cell culture. SEM

Similar cell culture and processing as described in Fig. 23, fixed 72h post-infection.

Original magnification: 20,000x. Final magnification: 32,000x.

Surface of a large syncytium. Phyllopodia are reduced to knobs or curved over the cell surface. The granular or scabrous aspect of the cell surface is more evident than in Fig. 38.

The infected cell

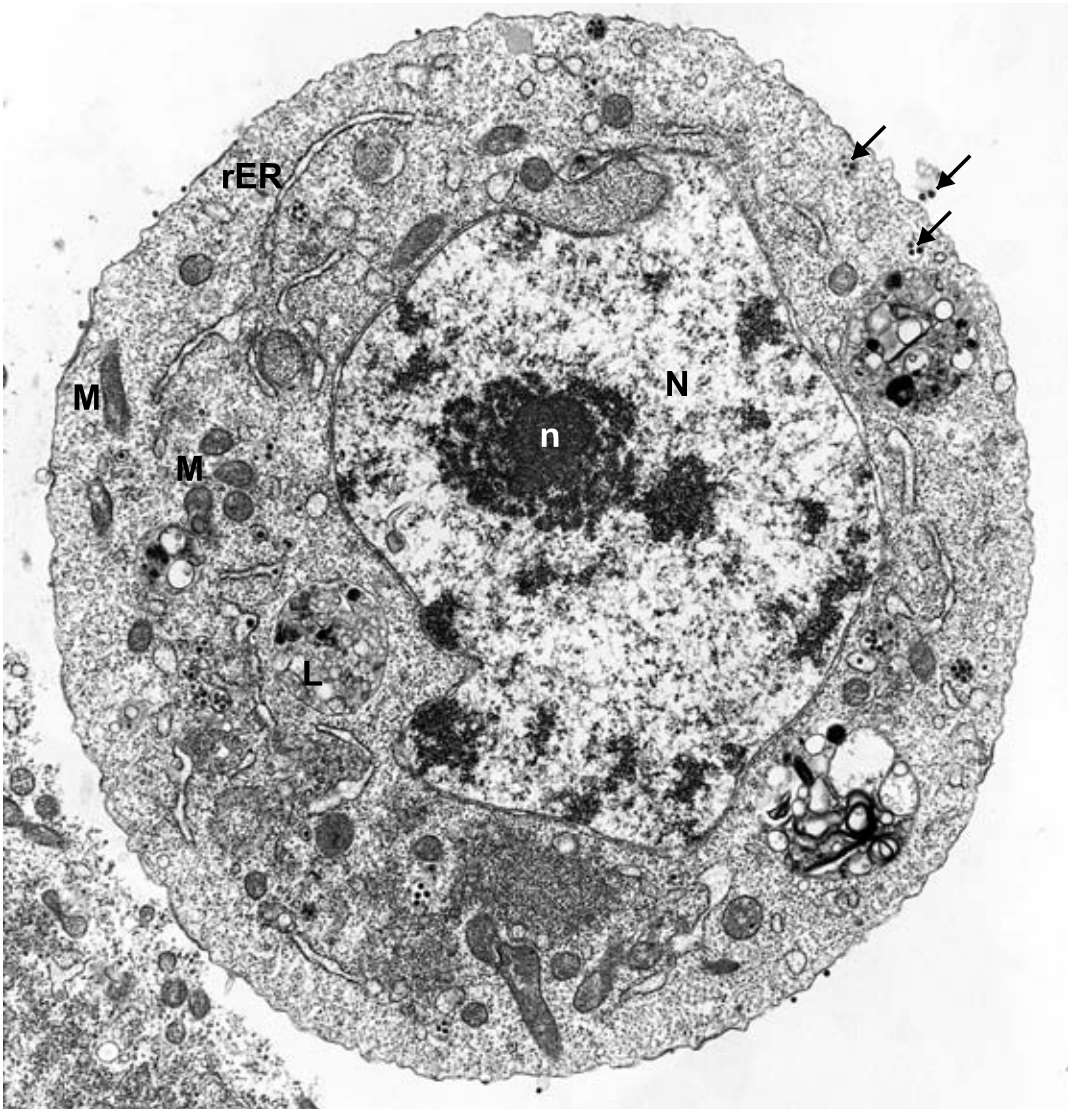


Fig. 40. Dengue-2 virus infected mosquito cell culture. TEM

A positive human dengue-2 serum and a dengue-1 human convalescent serum were mixed, incubated at 37°C for 30min. The infected cell monolayer was fixed 30sec post-infection with 1% phosphate-buffered glutaraldehyde. After washing in the same buffer, the cells were scraped off from the vial and post-fixed in 1% osmium tetroxide, washed, dehydrated in increasing concentration of acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss EM-900 transmission electron microscope.

Original magnification: 7,000x. Final magnification: 15,000x.

Darkly stained virus particles (arrows) may be observed outside, adsorbed to the mosquito cell membrane, and inside the cell cytoplasm. Groups of viral particles are enclosed in endocytic vesicles. Mitochondria, cell nucleus and nucleolus, rough endoplasmic reticulum and lysosomes can be distinguished.

The infected cell

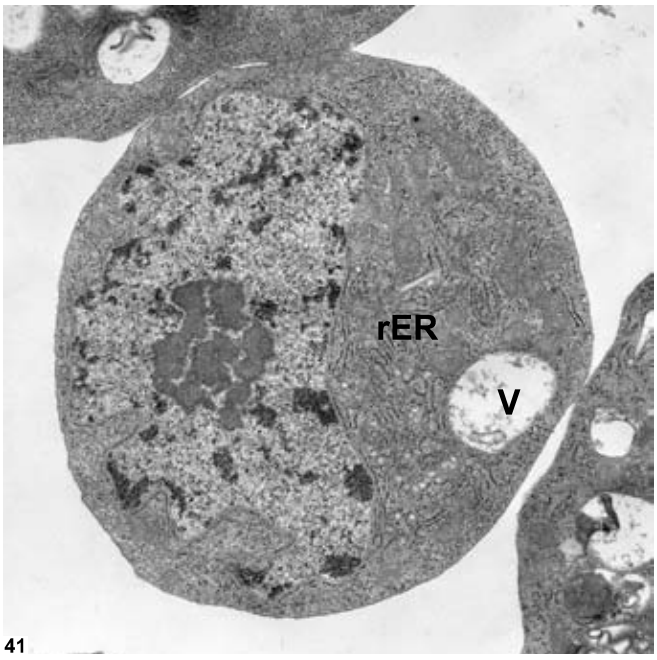


Fig. 41. Dengue-1 virus infected mosquito cell culture. TEM

The cell culture was fixed 5 days post-infection and standard processed as discribed in Fig. 40.

Original magnification: 5,000x. Final magnification: 13,000x.

The infected cell presents an excentrically displaced nucleus and viral morphogenesis is in progress, inside the cysterns of the rough endoplasmic reticulum.

The infected cell

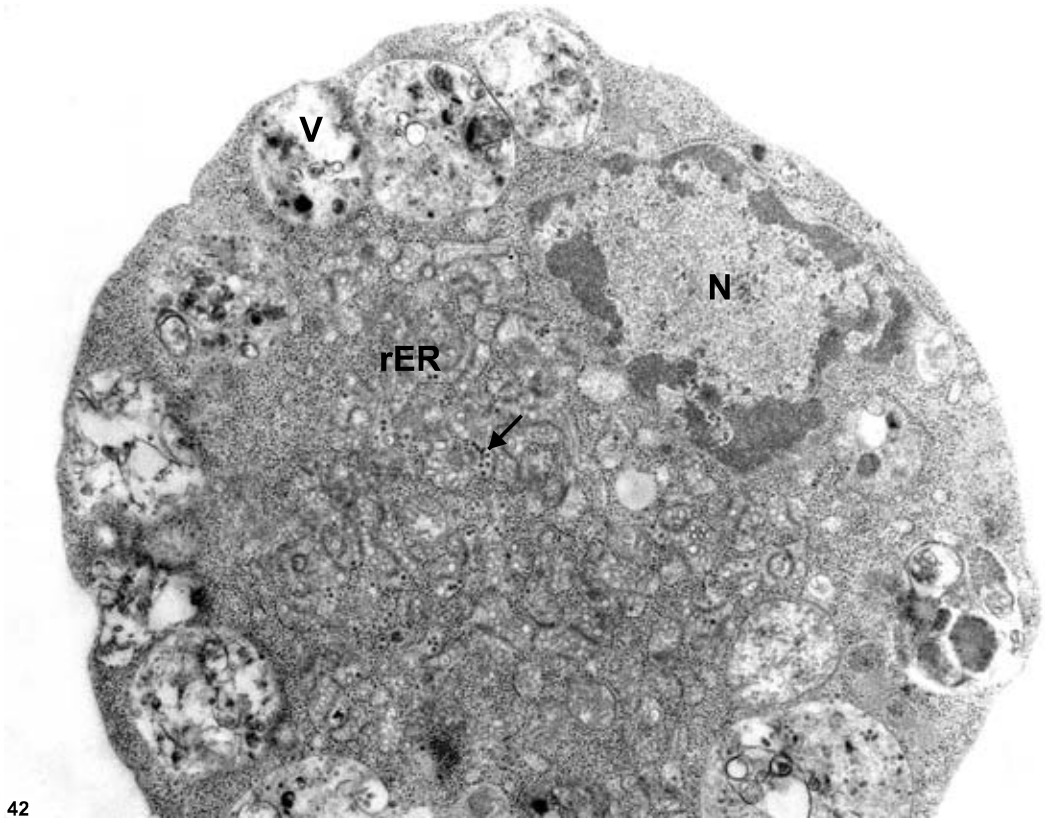


Fig. 42. Dengue-1 virus infected mosquito cell culture. TEM

The cell culture was fixed 4 days post-infection and standard processed as described in Fig. 40.

Original magnification: 10,000x. Final magnification: 17,500x.

The cell diameter increased post-infection. The nucleus was displaced to the cell periphery. Large vacuoles along the cell border start to open (compare with Fig. 35). The increased rough endoplasmic reticulum in the center of the cell contains numerous virus particles (arrow) (compare also with Figs. 16, 17 and 19).

The infected cell

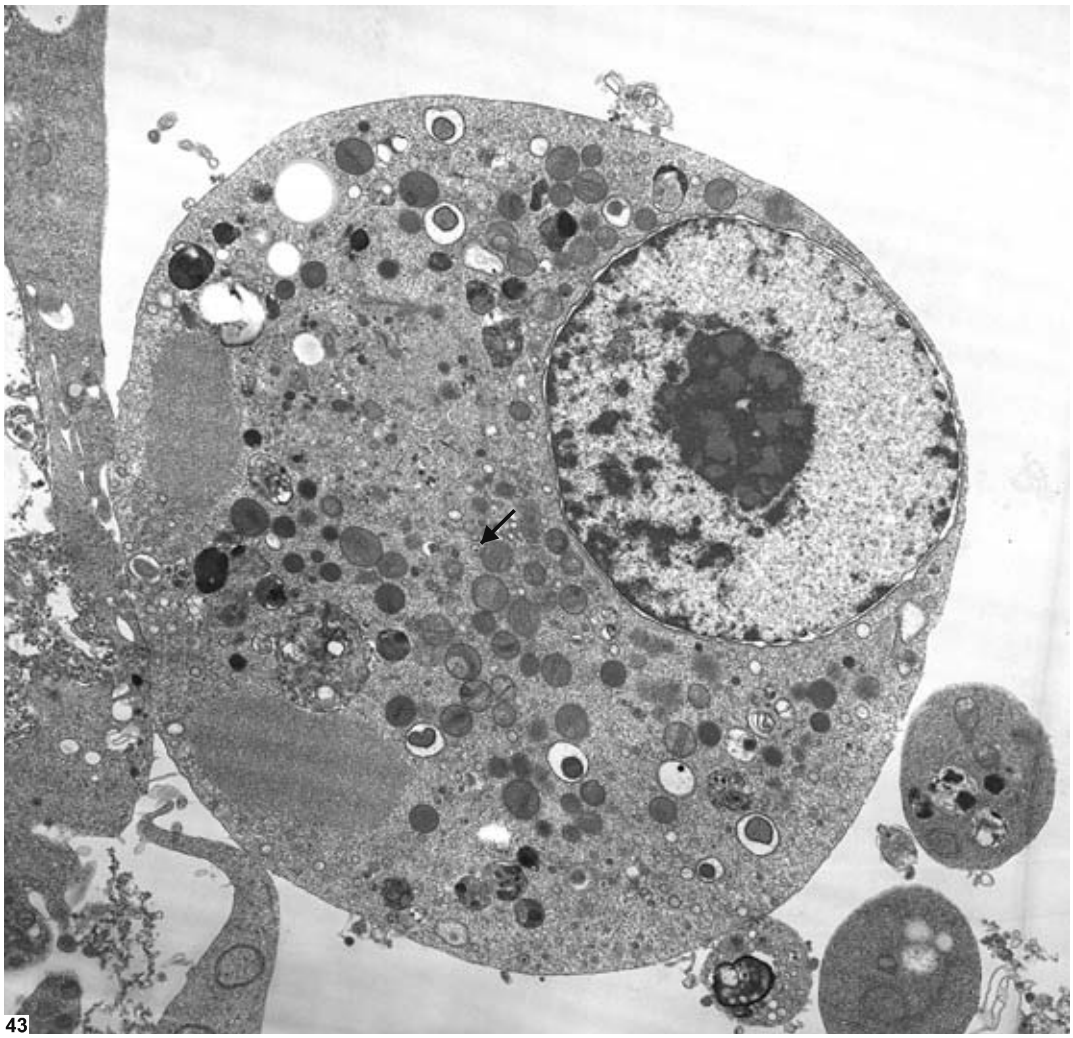


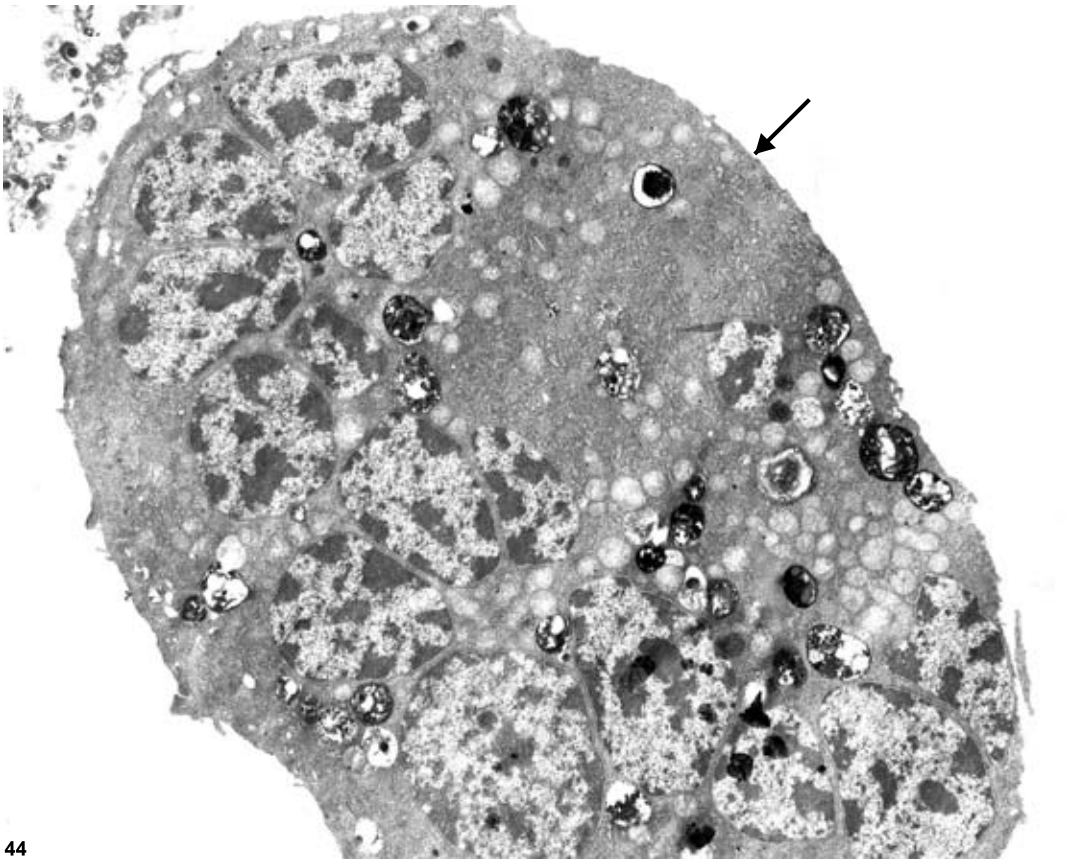
Fig. 43. Dengue-1 virus infected *Aedes pseudoscutellaris* (AP61) cell culture. TEM

The cell culture was fixed 6 days post-infection and standard processed as described in Fig. 40.

Original magnification: 4,400x. Final magnification: 10,000x.

The same aspect of a displaced cell nucleus as in infected clone C6/36 mosquito cells may be observed. Two large protein "pseudocrystals" are present at the left side cell periphery. Virus particles (arrow) are enclosed in cysterns of the rough endoplasmic reticulum.

The infected cell

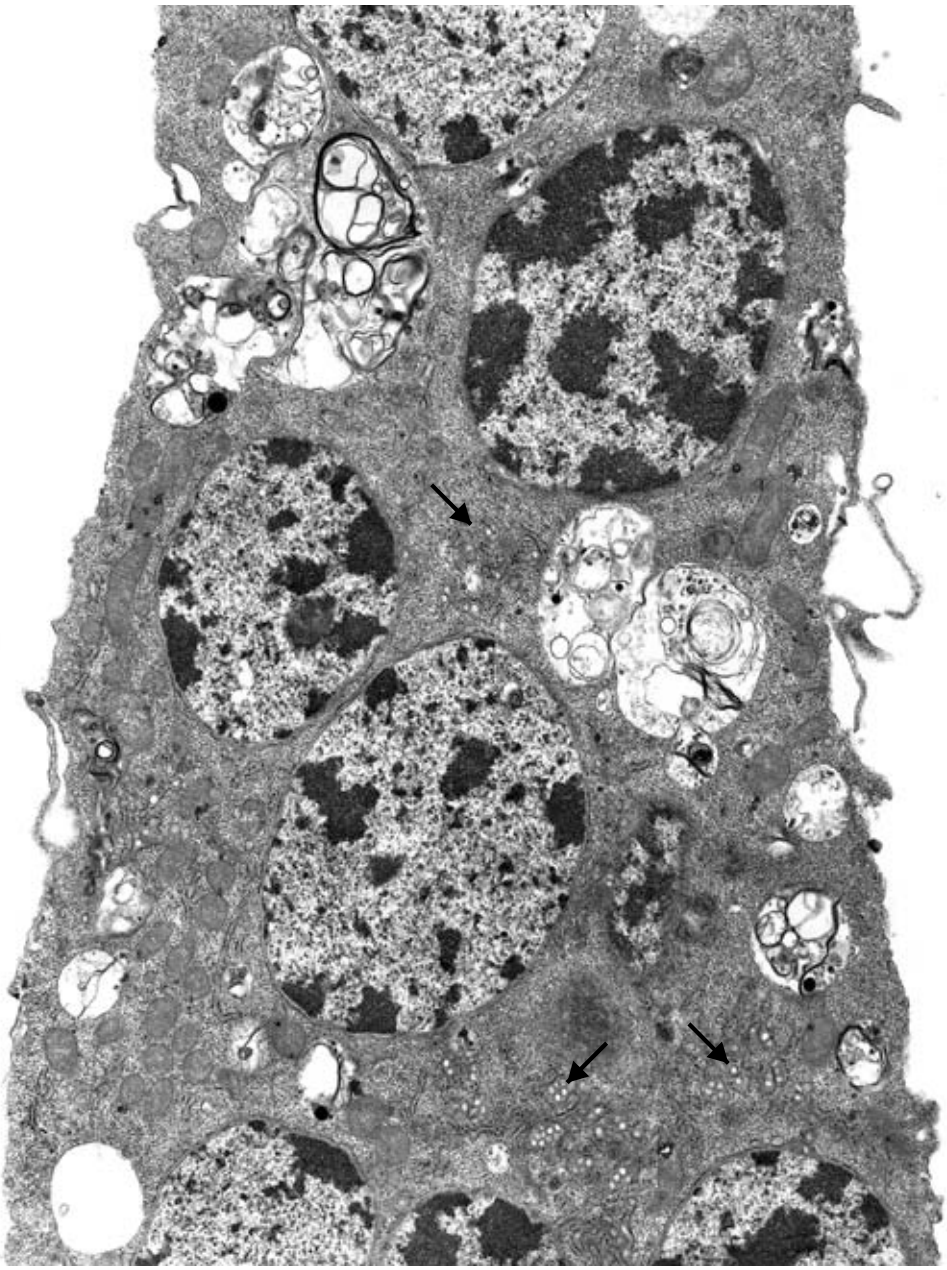


44

Fig. 44. Dengue-1 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection in cacodylate-buffered 1% glutaraldehyde, washed in acetate buffer and incubated in a solution of cerium chloride for one hour. After washing again with acetate and cacodylate buffers, the cells were fixed again with 3% glutaraldehyde, washed and scraped off from the vial. Post-fixation was done in 2% osmium tetroxide, dehydration in increasing concentrations of acetone and embedding in Epon (Robinson, 1985).
Original magnification: 3,150x. Final magnification: 6,000x.

This figure of low magnification shows a rounded syncytium with numerous nuclei, a well limited cytoplasmic area (arrow) where viral replication is in progress and dark stained lysosome content, indicative of acid phosphatase activity.



45

Fig. 45. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 5,000x. Final magnification: 10,000x.

Portion of an elongated syncytium with numerous nuclei and lysosomes. Areas of viral replication (arrows), located between these cell organells, are characterized by an increase of the rough endoplasmic reticulum and the presence of little unstained vesicles and dark stained virus particles inside the rER-cysterns. Very thin phyllopodia can be observed on the cell membrane.

The infected cell

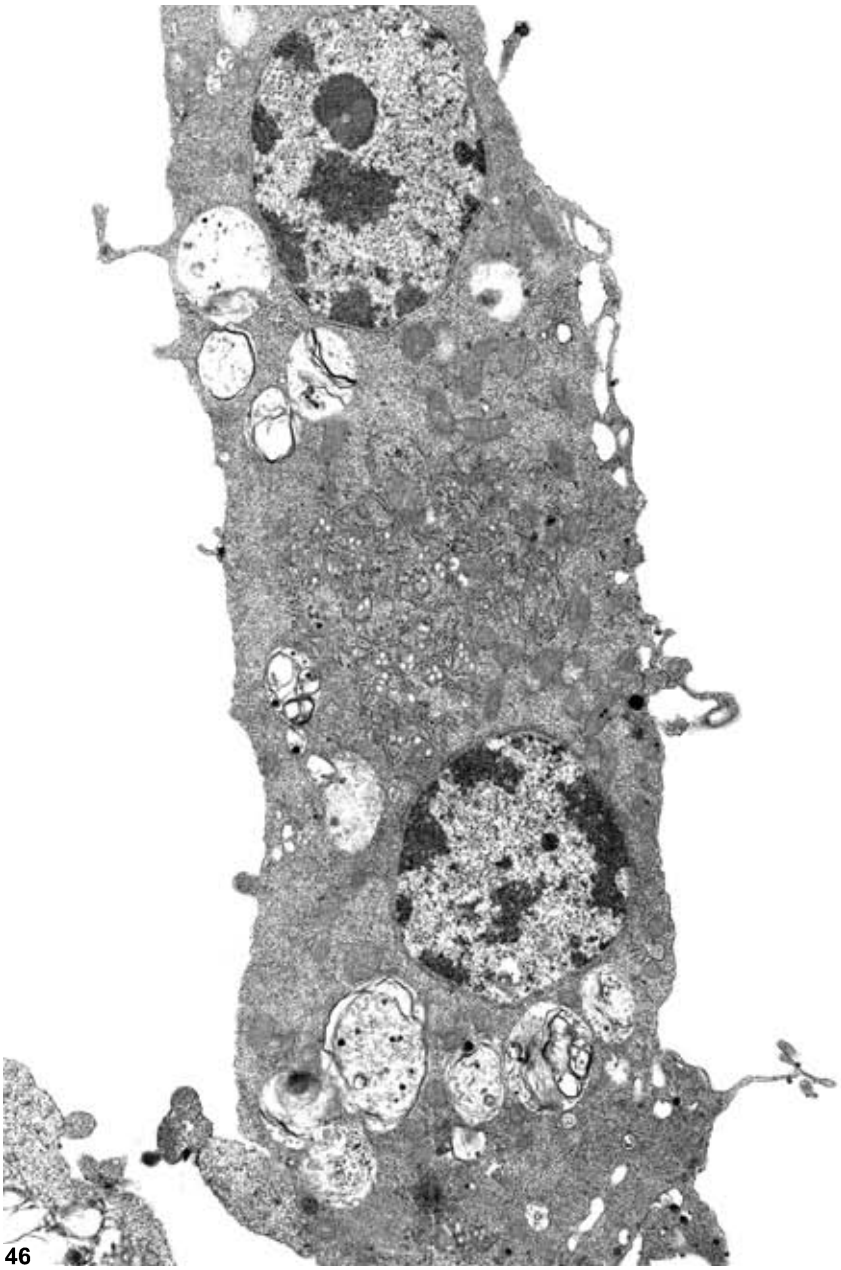


Fig. 46. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.
Original magnification: 5,000x. Final magnification: 10,000x.

This very long syncytium is characterized by a linear sequence of nuclei, lysosomes, vacuoles and areas of viral replication. Very thin phyllopodia are present on the cell surface, as illustrated also in Figs. 27, 34 and 45.

The infected cell

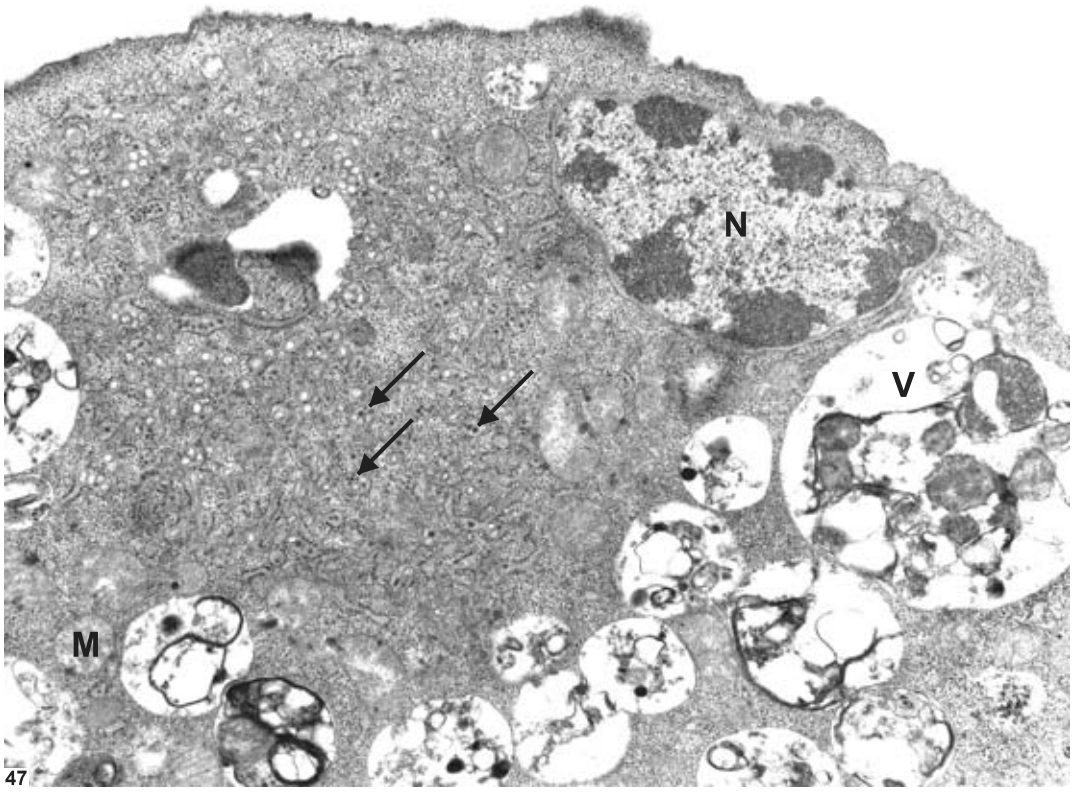


Fig. 47. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 8,000x. Final magnification: 16,000x.

An enlarged area of viral replication inside a syncytium. The nucleus is displaced to the cell periphery. Lysosomes and vacuoles containing dark material are located around this area. Mitochondria are not so evident. The area of viral replication is characterized by the increase of rough endoplasmic reticulum network and cysterns, where dark stained virus particles (arrows) and light smooth membrane structures are detectable inside.

The infected cell

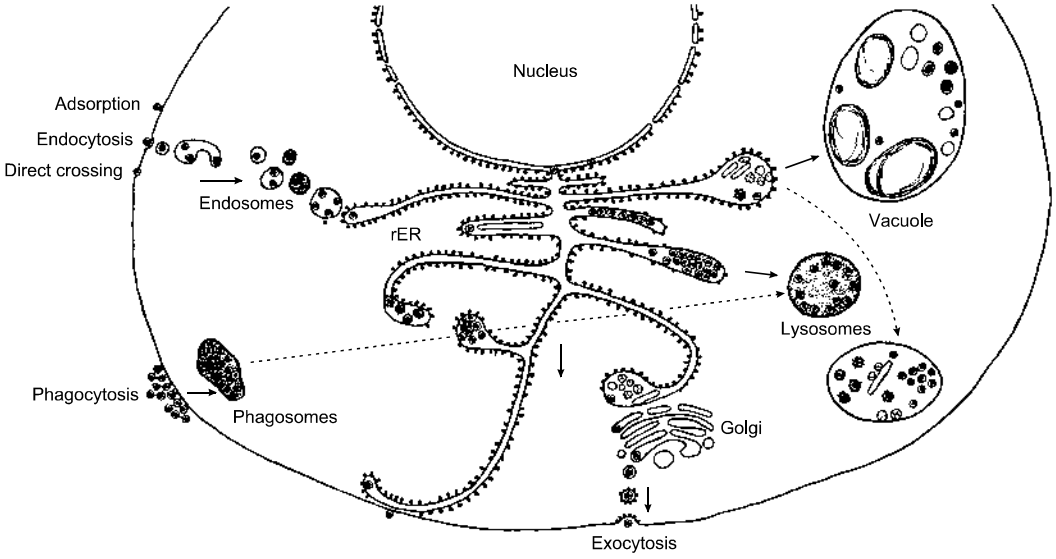


Fig. 48. A model of dengue virus morphogenesis

The active phase of this event is constituted of virus particle adsorption to the cell membrane, its entry into the cell using three ways of penetration (endocytosis, phagocytosis and direct penetration), the replication process itself and the appearance of new virus particles and smooth membrane structures inside the rough endoplasmic reticulum, followed by virus transport throughout the Golgi complex and finally exocytosis.

The passive phase comprises virus inactivation inside lysosomes and its processing inside vacuoles. (from Barth, 1992, modified)

These different steps of viral life and death are illustrated in the following plates.

Adsorption

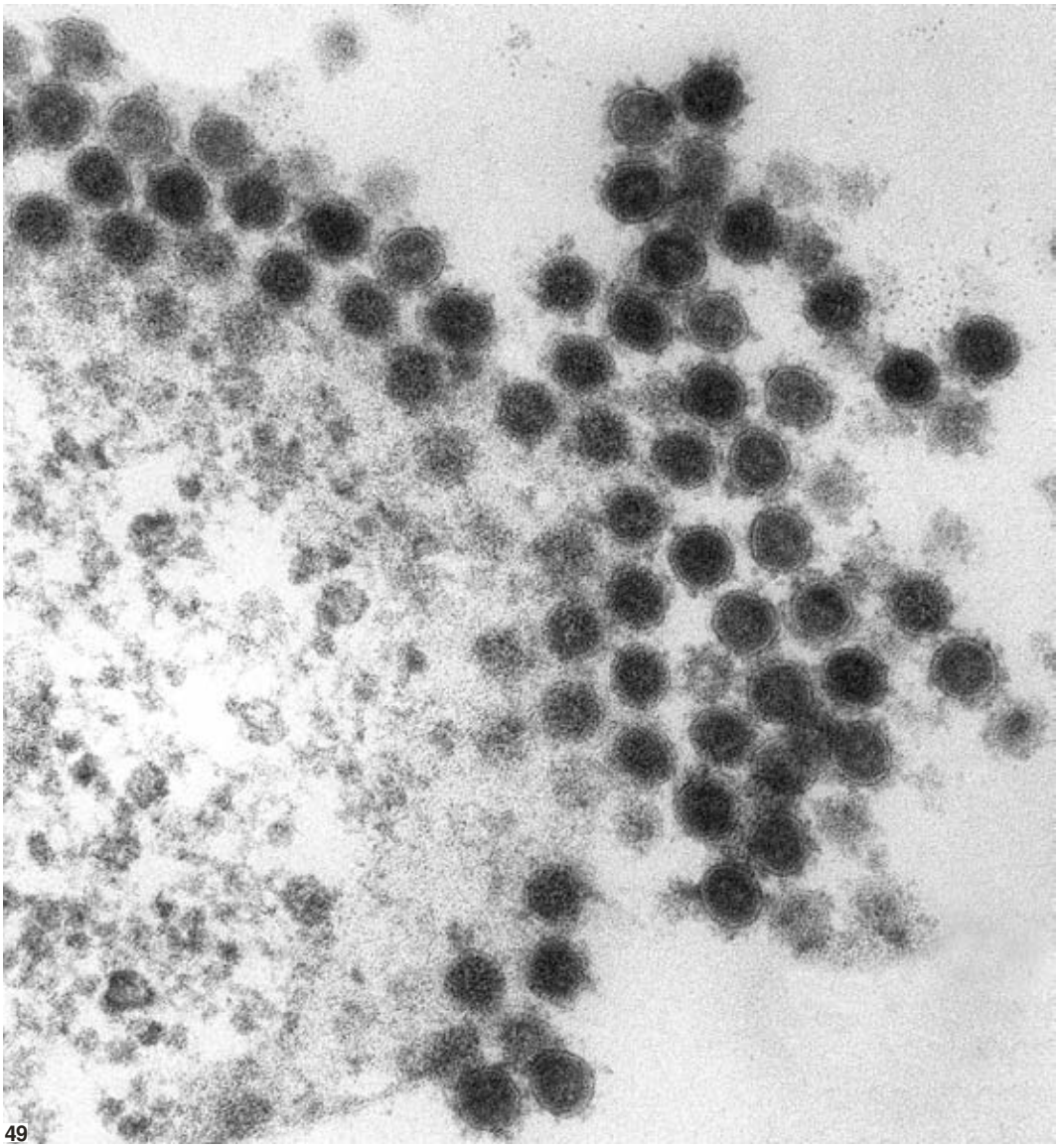


Fig. 49. Dengue-2 infected mosquito cell culture. TEM

A positive human dengue-2 serum and a dengue-1 human convalescent serum were mixed and incubated at 37°C for 30min. The cell culture was fixed 30min post-infection and standard processed as described in Fig. 40.

Original magnification: 50,000x. Final magnification: 130,000x.

Viral particles are joined by antibodies from the human dengue-1 convalescent serum and adsorbed to the cell membrane. This frontal view is a nearly parallel section to the plasma membrane surface. Irregularly shaped ribosomes are dispersed inside the cytosol at the left side of the figure. Each virus particle presents an electron-dense core surrounded by a triple shelled unit membrane (dark, light, dark), where viral proteins are insered and antibodies are linked to. Each virus particle
shows a fuzzy coat.

Penetration: endocytosis

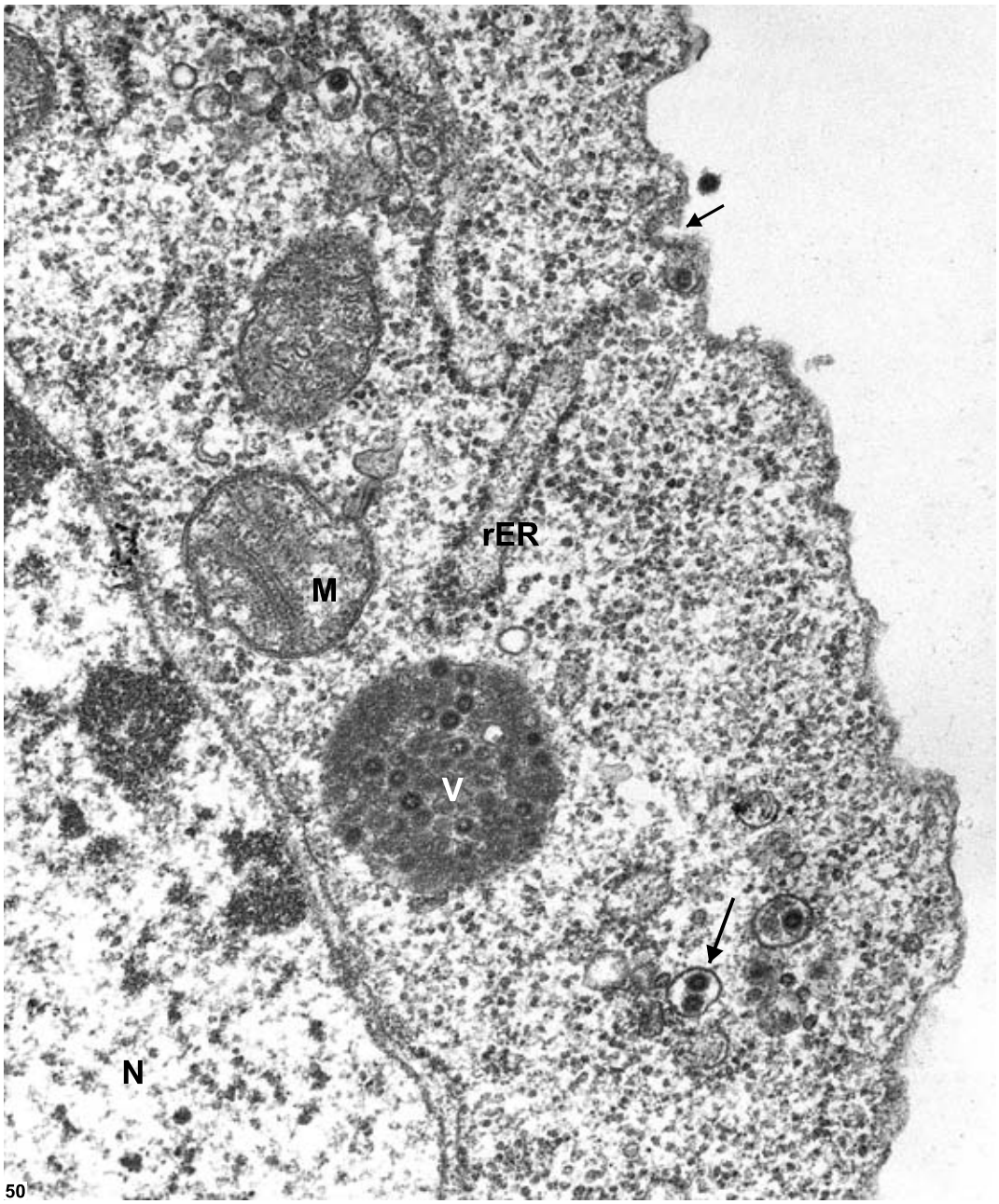


Fig. 50. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 40.
Original magnification: 20,000x. Final magnification: 48,000x.

A single virus particle outside the cell membrane may be observed at the upper right side of the figure; it is adsorbed by antibodies, that do not appear in this section, to the cell membrane. Below, a starting endocytic invagination of the cell membrane was formed (small arrow) and a virus containing endocytic vesicle is nearly closed. Inside the cell cytoplasm there are numerous virus containing endocytic vesicles (long arrow), cysterns of the rough endoplasmic reticulum, a phagocytic vesicle with numerous virus particles inside, mitochondria and the cell nucleus.

Penetration: endocytosis

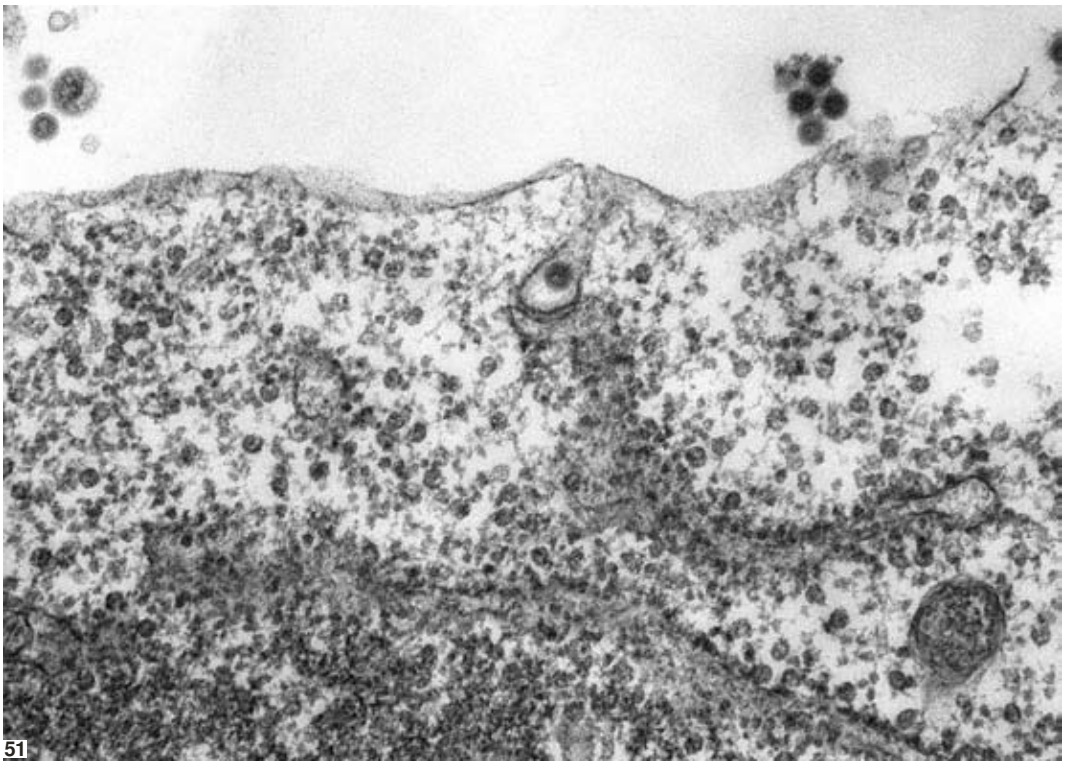


Fig. 51. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 49.

Original magnification: 30,000x. Final magnification: 63,000x.

Clusters of virus particles are located outside the cell. One virus particle penetrates the cell by aid of a endocytic smooth membrane coated vesicle (endocytic vesicle). A long channel is formed, connecting the plasma membrane and the endocytic vesicle. The cysterns of the rough endoplasmic reticulum are densely coated with ribosomes.

Penetration: endocytosis

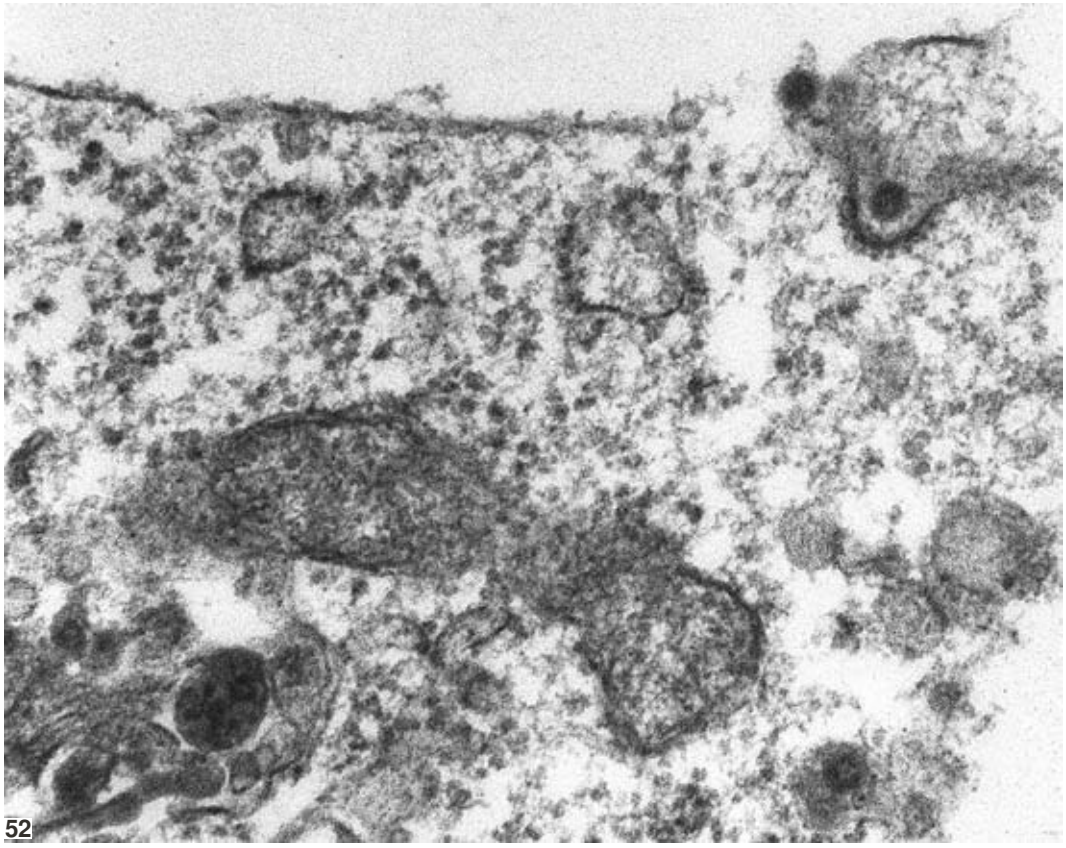


Fig. 52. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 49.

Original magnification: 30,000x. Final magnification: 72,000x.

A single virus particle is attached to the plasma membrane. Another virus particle is engulfed by a probably clathrin coated endocytic vesicle. Below (arrow), a virus particle inside an endosomial vesicle is ready for viral genome discharge into the cytosol, starting replication.

Penetration: endosomes

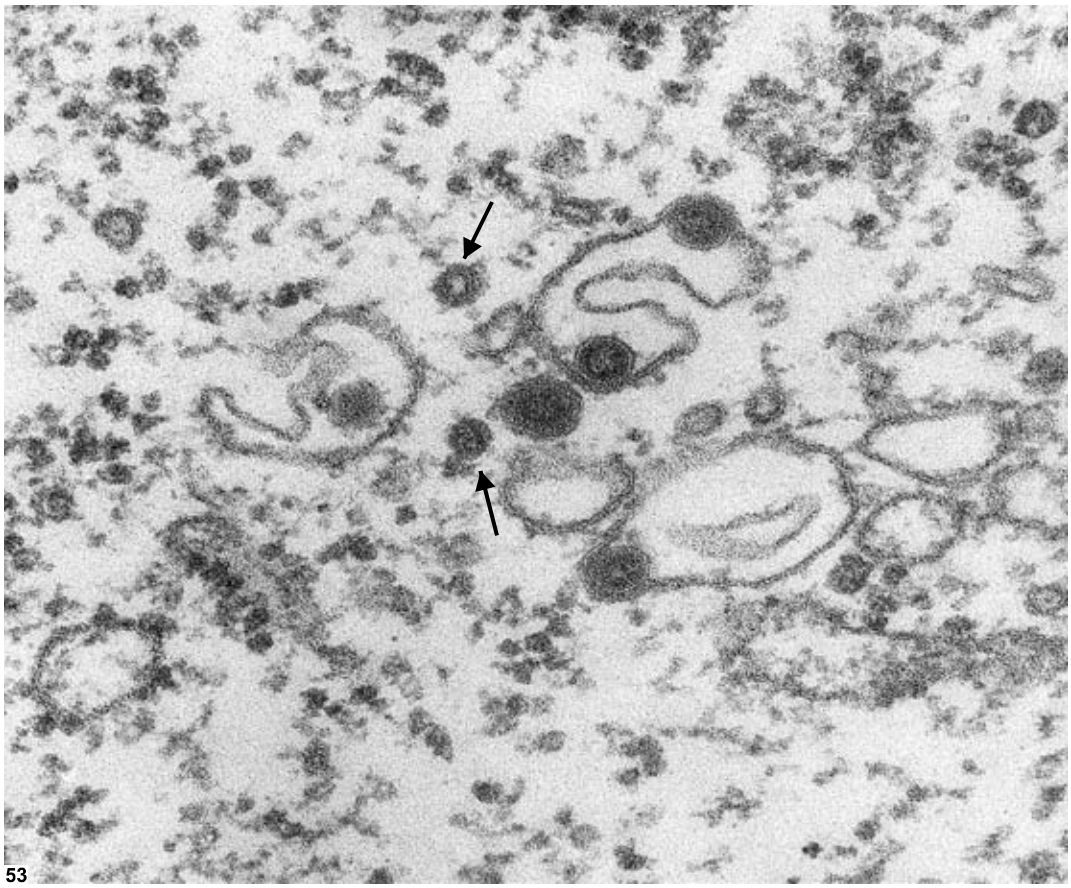


Fig. 53. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 40.
Original magnification: 50,000x. Final magnification: 120,000x.
Multiple shaped endosomes containing dengue virus particles. Fusion of viral envelopes with endosome membranes is starting and viral cores (arrows) are released into the cell cytoplasm.

Penetration: endosomes

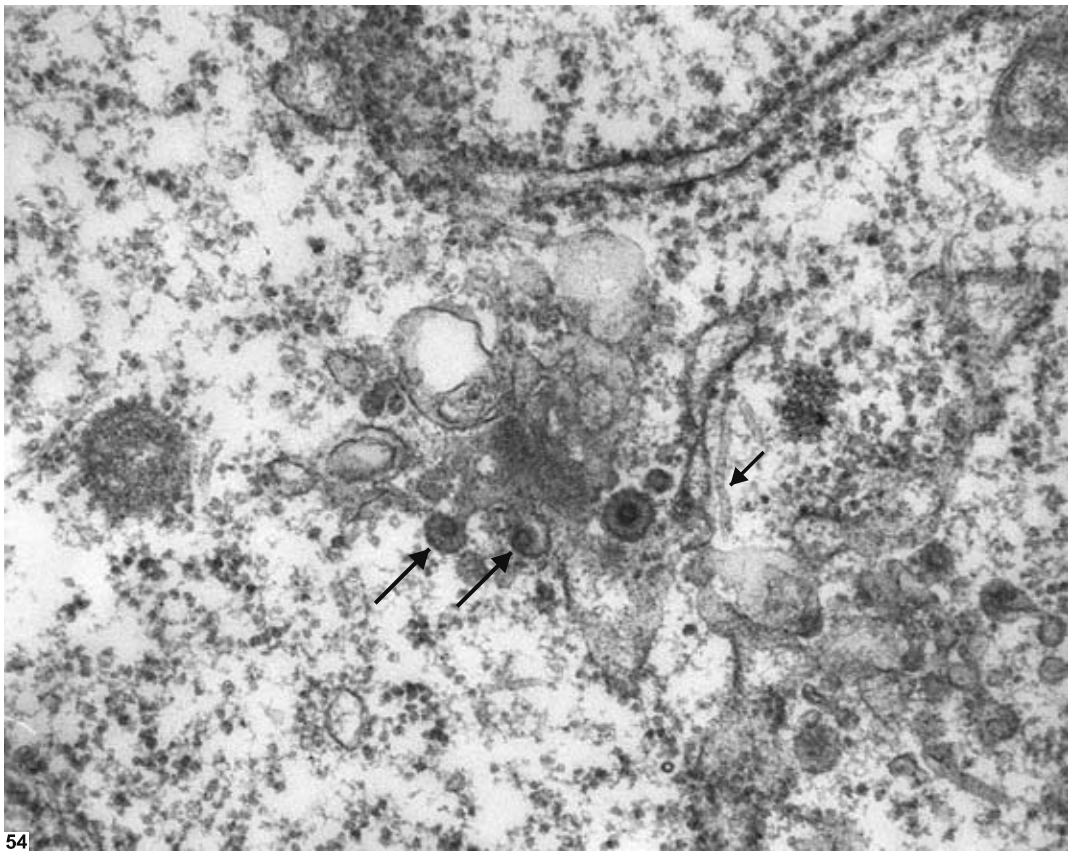


Fig. 54. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 40.

Original magnification: 30,000x. Final magnification: 58,000x.

The endosomial network in the center of the figure shows two dengue virus particles (large arrows) enclosed each one in an endosomial vesicle. Microtubules (small arrow) for vesicle transport are present.

Penetration: phagosomes

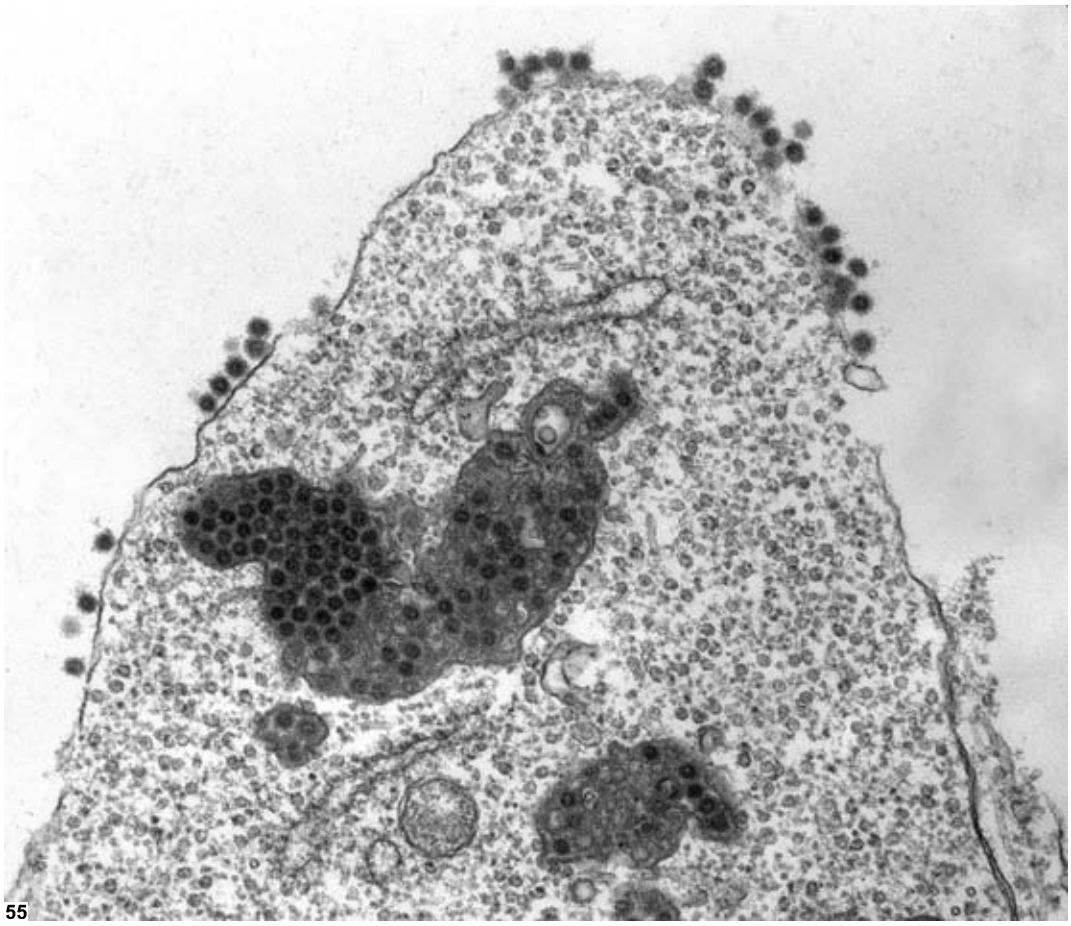


Fig. 55. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 49.

Original magnification: 20,000x. Final magnification: 40,000x.

Dengue virus particles are adsorbed to the cell membrane. Inside the cell, a large phagosome engulfed numerous virus particles; this compartment will be transformed into a lysosome and further in a vacuole, by lowering the pH values. These virus particles shall be inactivated.

Penetration: phagosomes

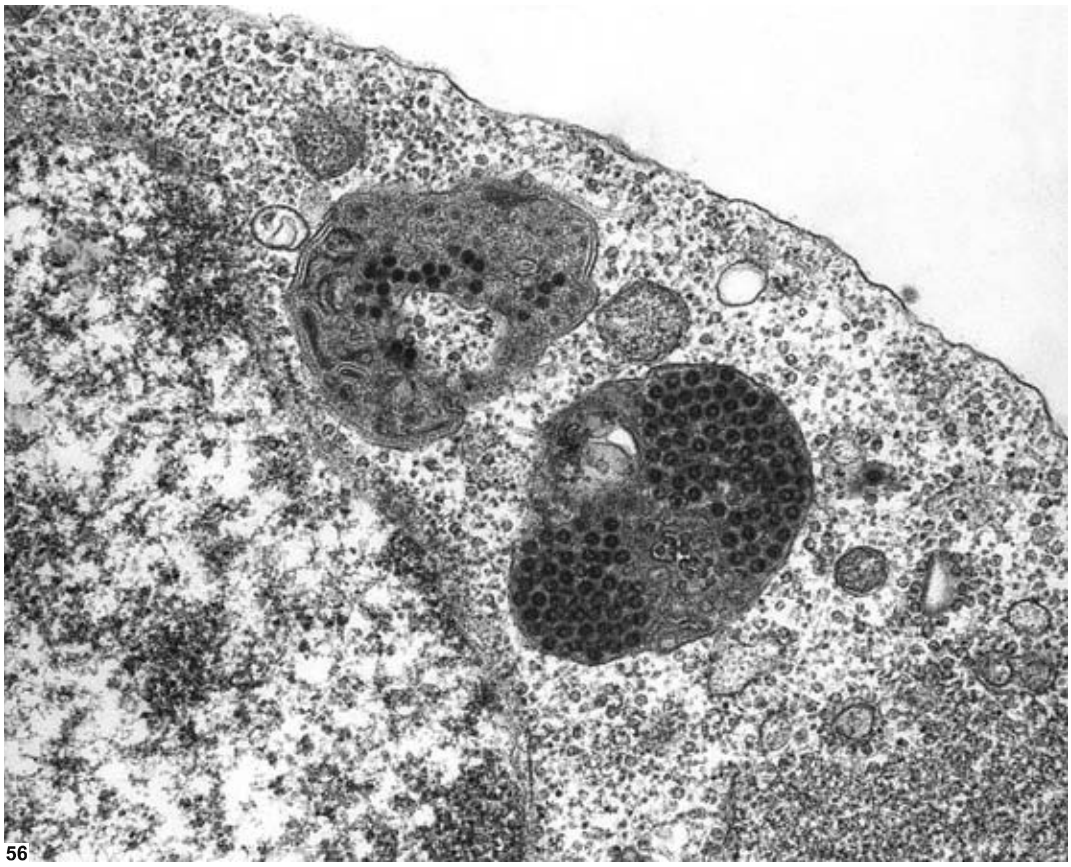


Fig. 56. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 40.

Original magnification: 20,000x. Final magnification: 38,000x.

Two large phagosomes present a variable number of incorporated virus particles. A virus particle containing little endosome is present in the right side of the figure.

Penetration: cell fusion

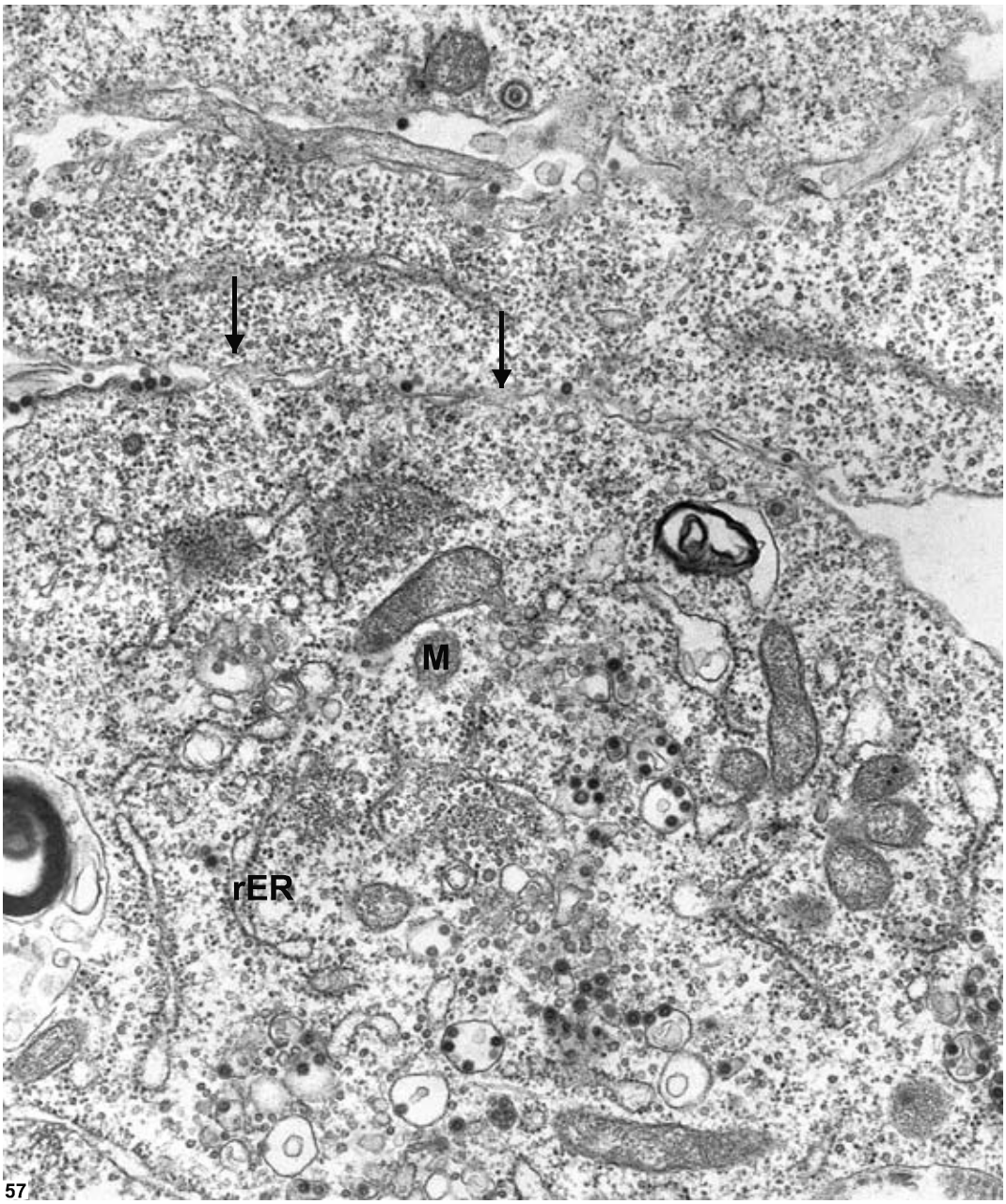


Fig. 57. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 40.

Original magnification: 12,000x. Final magnification: 31,000x.

Two cells start fusion “from within” mediated by dengue virus particles. At different points (arrows) the fusion is effective, while beside these, the cell membranes are yet present. In the middle and lower parts of the figure, numerous endosomes and cysterns of the endosomial network contain several virus particles. Mitochondria and cysterns of the rough endoplasmic reticulum may be recog
nized. A clathrin coated endocytic vesicle is located near to the cell membrane in the upper part of the figure. The several cell to cell connections established are in correspondence with the scanning electron micrograph in Fig. 30.

Penetration: cell fusion

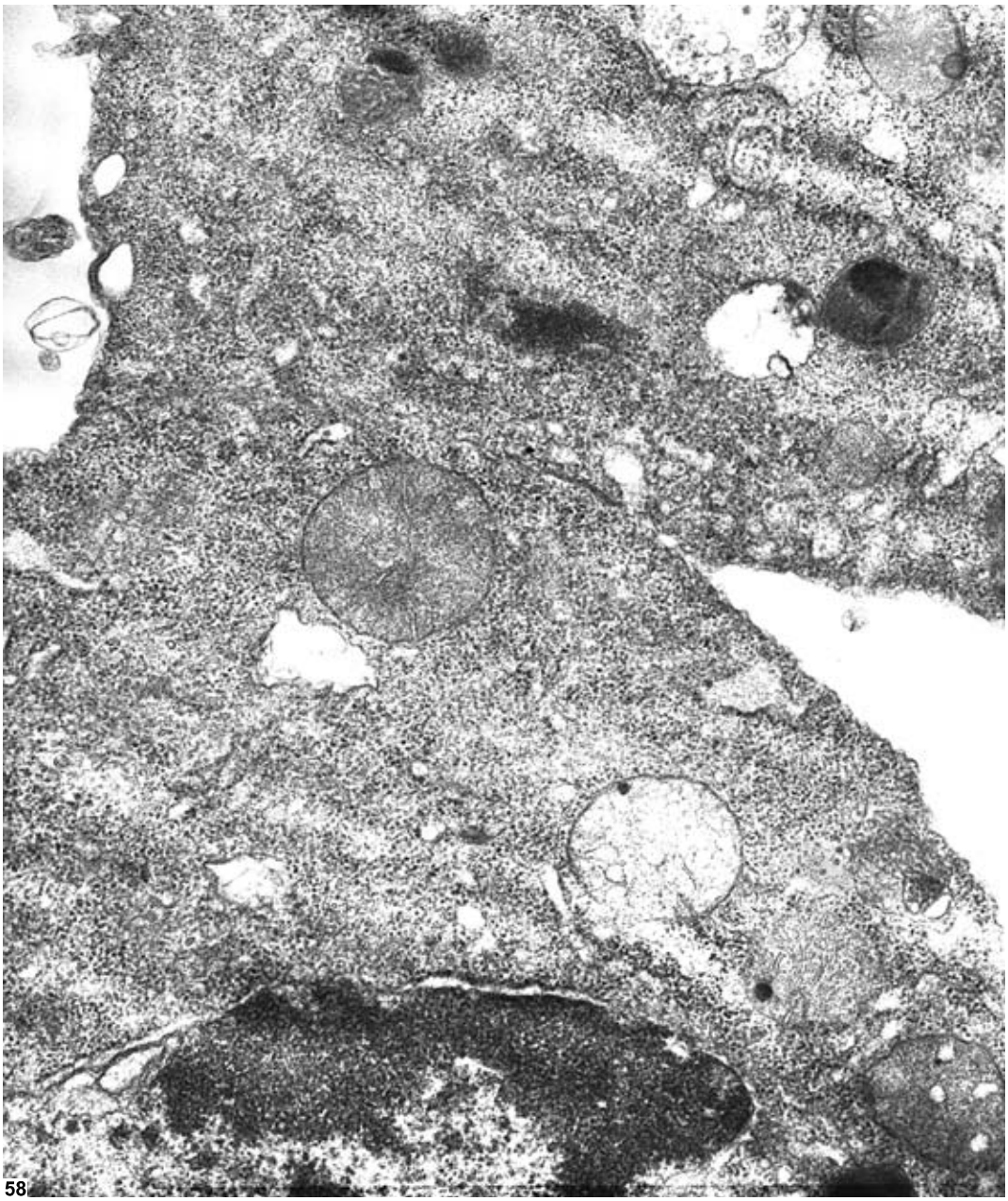


Fig. 58. Dengue-1 virus infected mosquito cell culture. TEM

The cell culture was fixed 6 days post-infection and standard processed as described in Fig. 40.

Original magnification: 12,000x. Final magnification: 31,000x.

A large connection between two cells was established, mediated by the presence of dengue virus particles, so that cell organells can pass from one to another. The formation of a syncytium is starting.

Transport: edosomial network

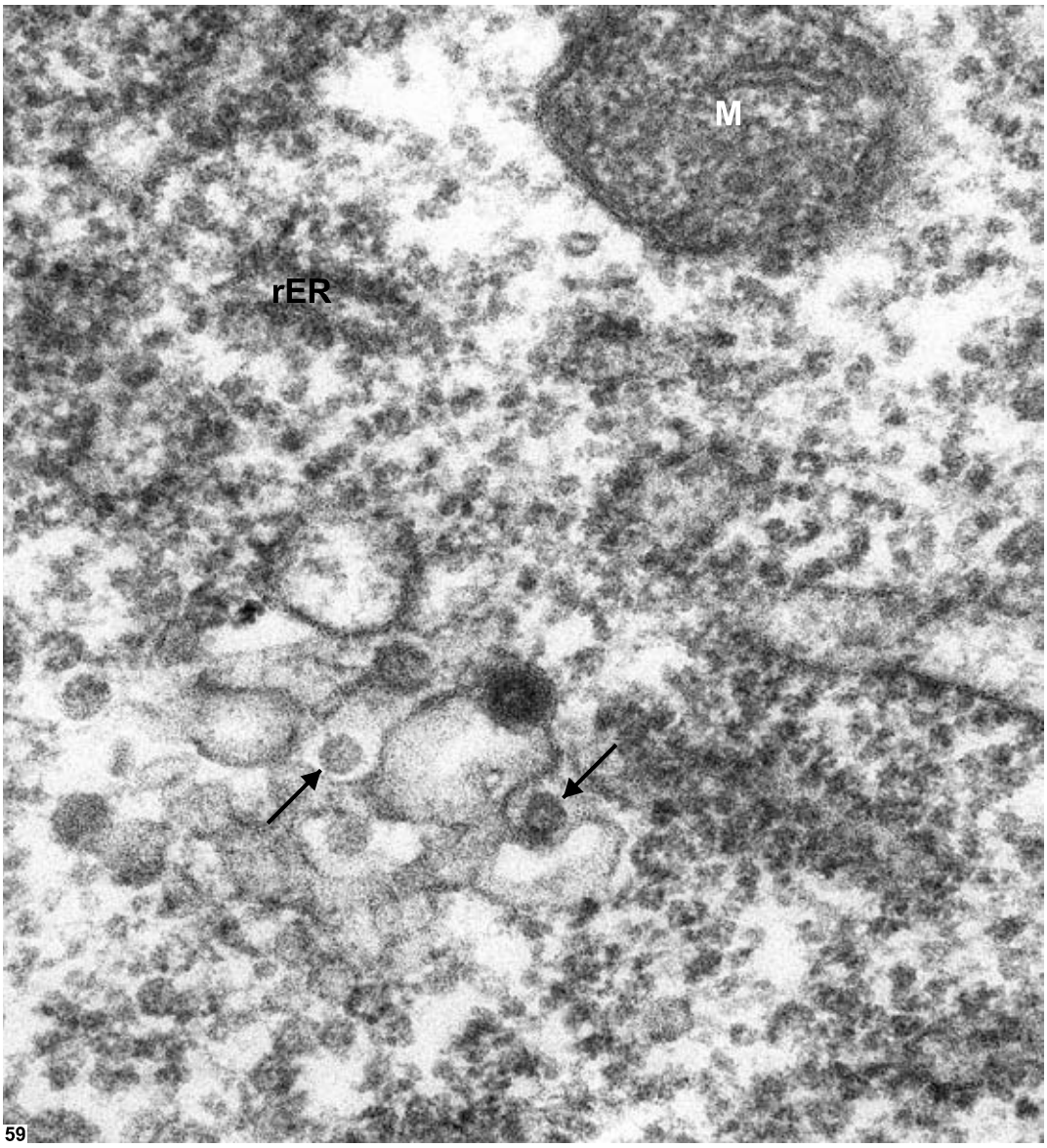


Fig. 59. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 40.

Original magnification: 50,000x. Final magnification: 100,000x.

A portion of the endosomial network, rough endoplasmic reticulum and one mitochondrion are represented. One dark stained virus particle is fusing with the membrane of an endosome. Several viral nucleocapsids (arrows) are already delivered into the cell cytoplasm.

Transport: endosomial network

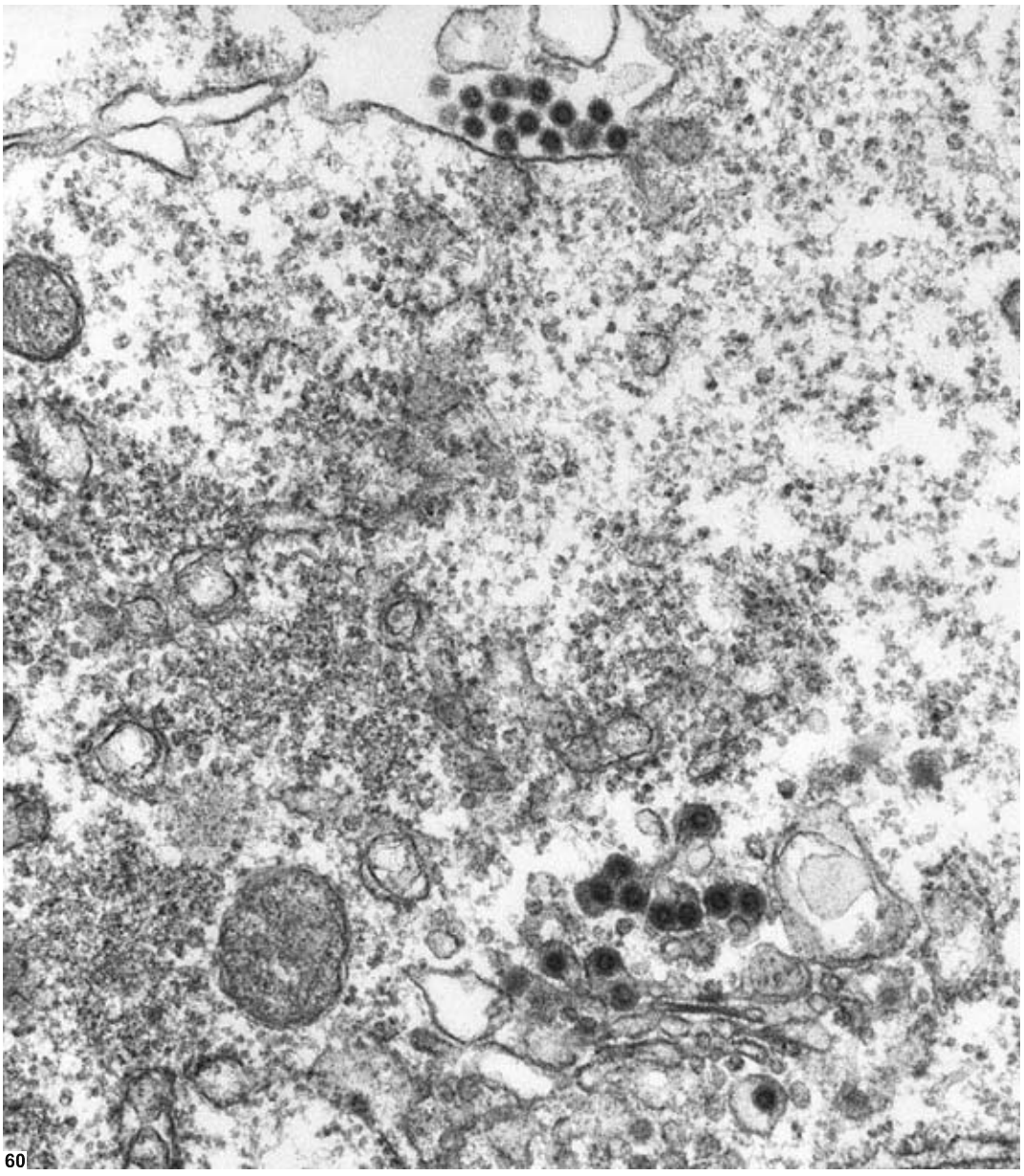


Fig. 60. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 49.

Original magnification: 20,000x. Final magnification: 50,000x.

A group of antibody linked virus particles is adsorbed to the cell membrane in the upper part of the figure. In the lower right side of the figure, virus particles stay inside cysterns of the endosomial network and viral nucleocapsids are delivered into the cell cytoplasm.

Replication

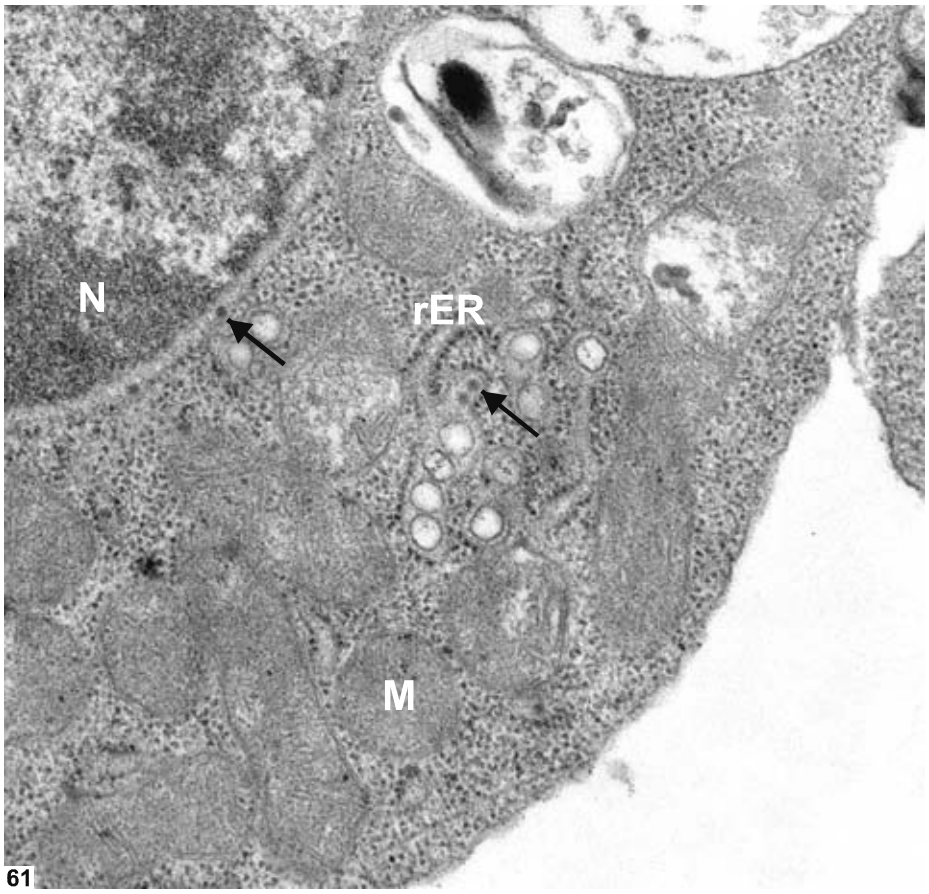


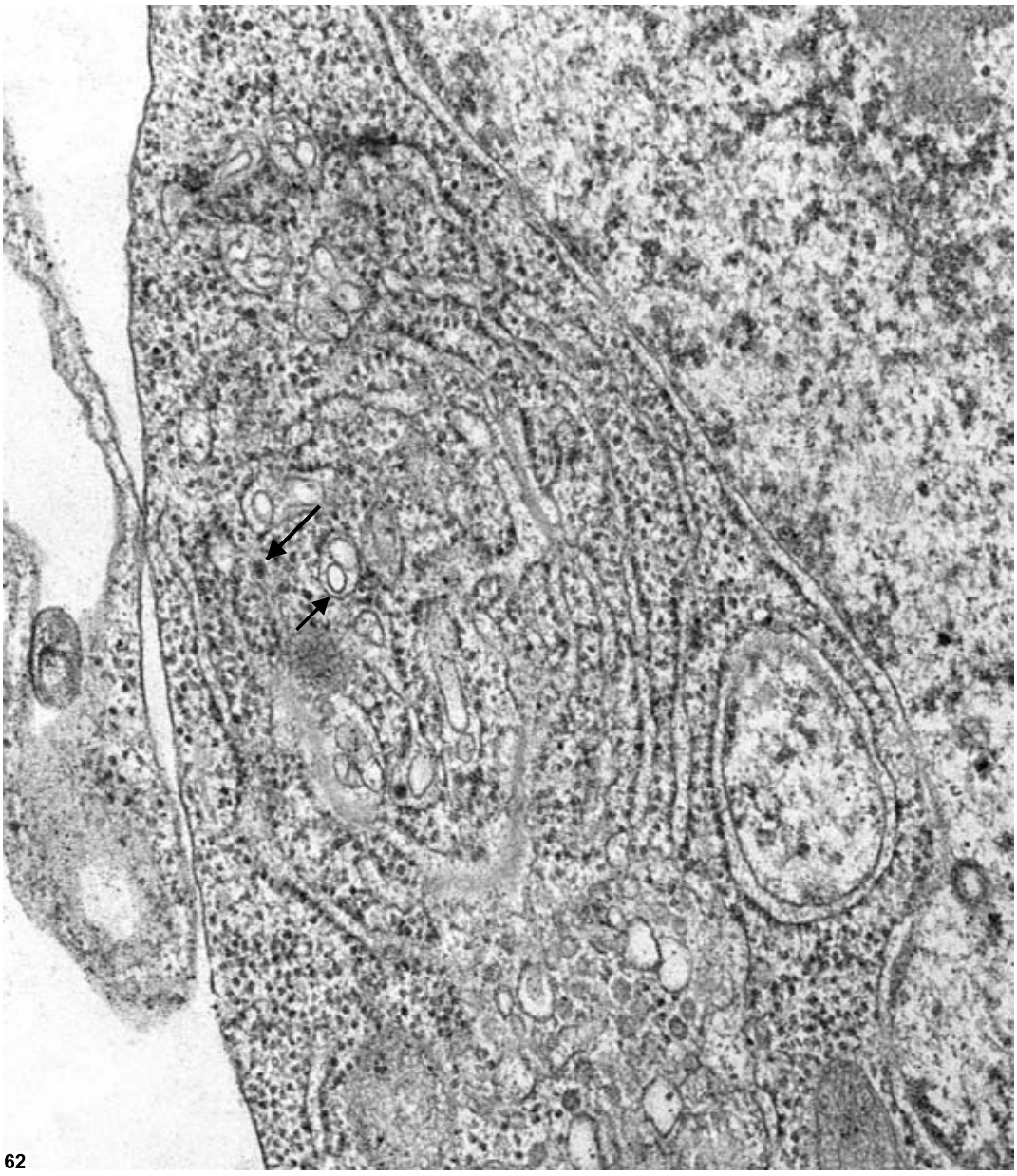
Fig. 61. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 16,500x. Final magnification: 40,000x.

Early stage in viral replication. The cell nucleus is not yet displaced from the central region of the cell. Mitochondria are abundant and the rough endoplasmic reticulum presents the first virus particles (arrows) and smooth membrane structures (light vesicles) inside. Virus particles occur also inside the nuclear envelope, that is considered as a portion of the rough endoplasmic reticulum (Figs. 48, 68-70).

Replication



62

Fig. 62. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40. Ultrathin sections were over-stained using 5% aquous uranyl acetate for 2min and treated with 0.2M EDTA during 20-30min, in order to clear the DNA-containing structures. In sequence, the sections were stained only with lead citrate (Bernhard, 1969).

Original magnification: 20,000x. Final magnification: 52,000x.

Early stage in viral replication. Concentrically disposed cysterns of the rough endoplasmic reticulum contain few viral particles (long arrow) and smooth membrane structures (small arrow). The low contrast of nuclear DNA, due to the extraction of the uranyl stain by EDTA, are in opposition to the dark stained RNA of ribosomes and virus particles.

Replication

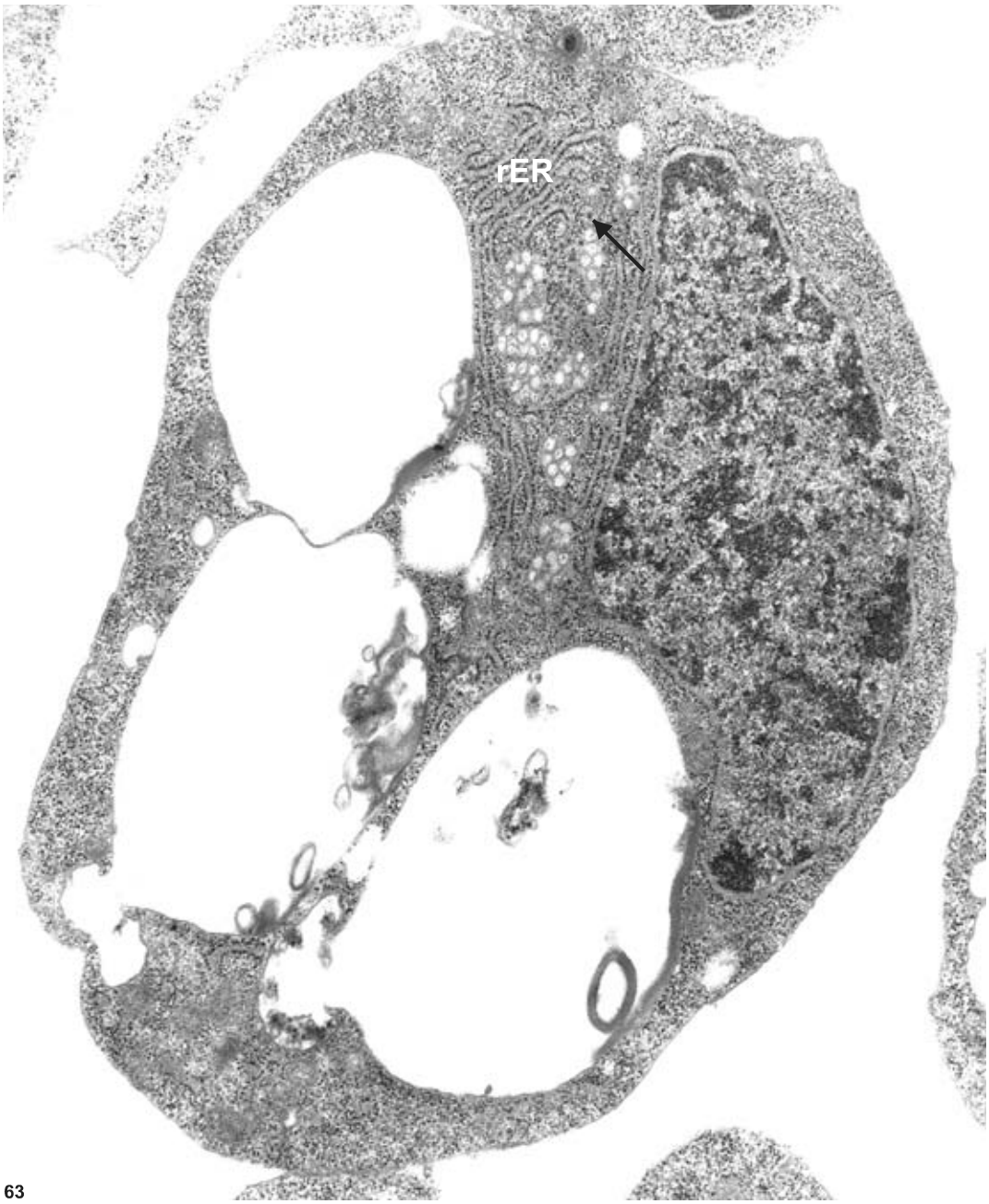


Fig. 63. Dengue-1 virus infected mosquito cell culture. TEM

The cell culture was fixed 2 days post-infection and standard processed as described in Fig. 40.

Original magnification: 10,000x. Final magnification: 24,000x.

The increasing of concentrically disposed cysterns of the rough endoplasmic reticulum and of cell vacuolization displaced the cell nucleus to the cell border. Smooth membrane structures (light dots) are more frequent inside the rough endoplasmic reticulum; few viral particles (arrow) can be detected.

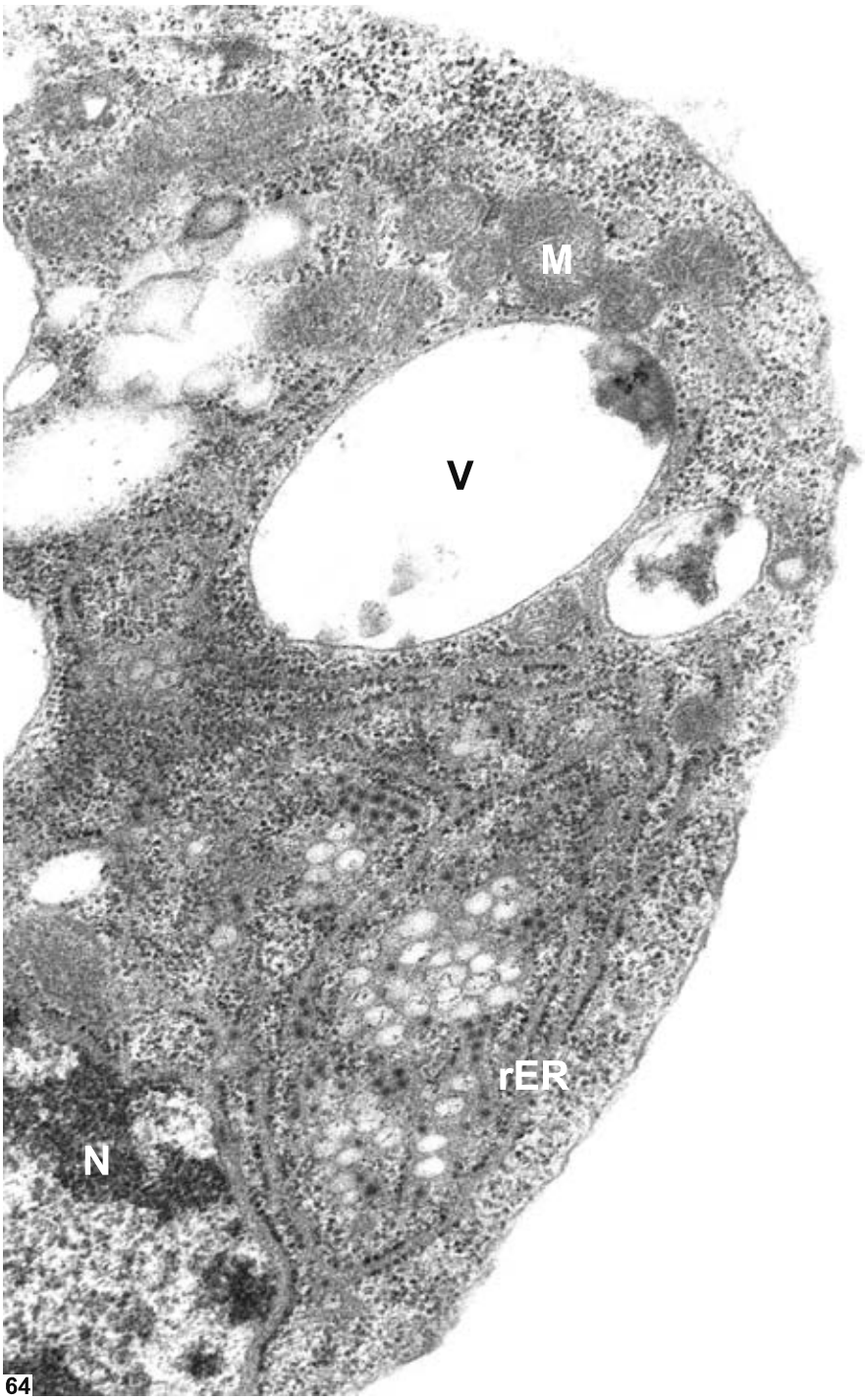


Fig. 64. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 63.
Original magnification: 16,500x. Final magnification: 45,000x.
A higher magnification of similar structures presented in Fig. 63. The expanded cysterns of the rough endoplasmic reticulum contain numerous viral particles (dark dots) and smooth membrane structures (light vesicles).

Replication

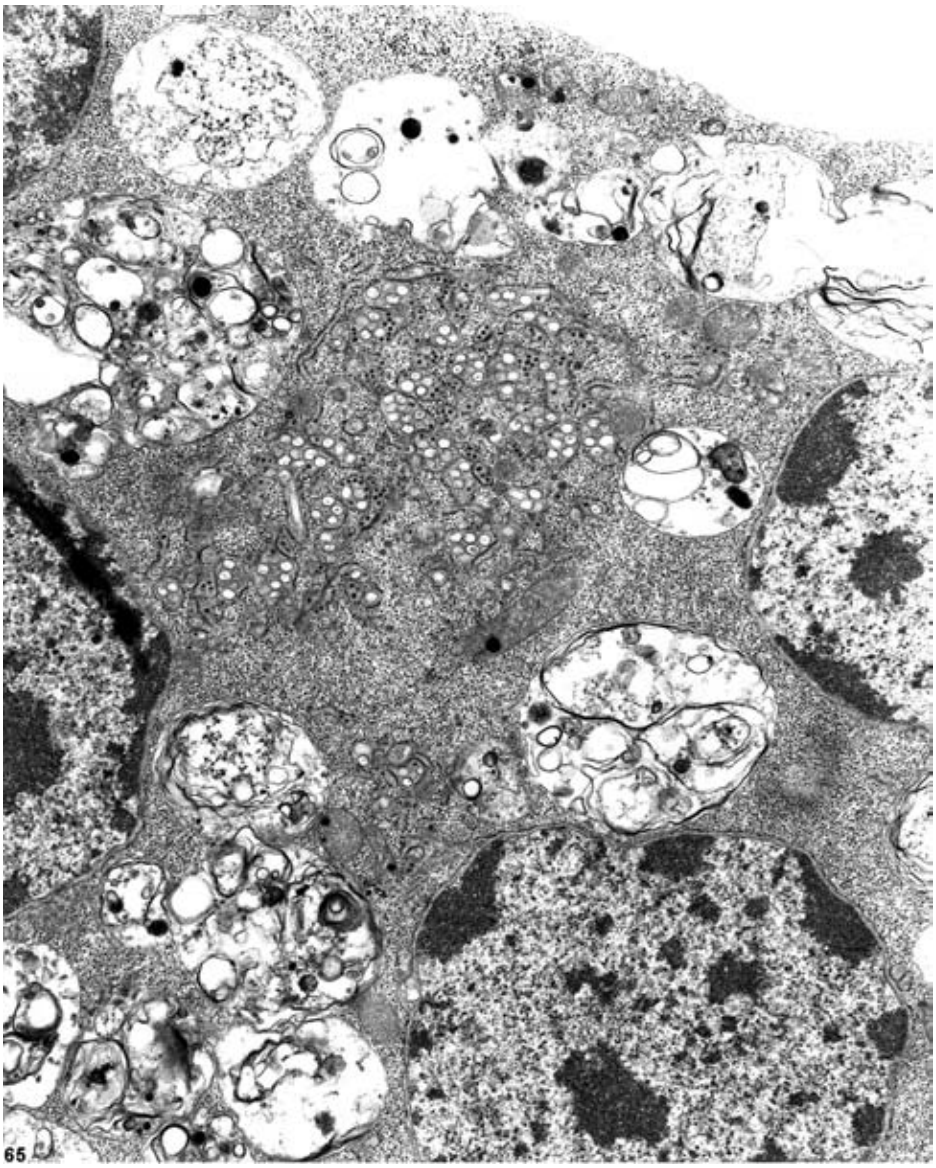


Fig. 65. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 8,000x. Final magnification: 16,000x.

Syncytium. The region of viral replication is located concentrical between randomly displaced nuclei and vacuoles. Numerous viral particles (little dark dots) and smooth membrane structures (light vesicles and tubules) can be detected inside the cysterns of the rough endoplasmic reticulum. The present aspect of a syncytium was also represented using confocal microscopy in Figs. 16 and 17.

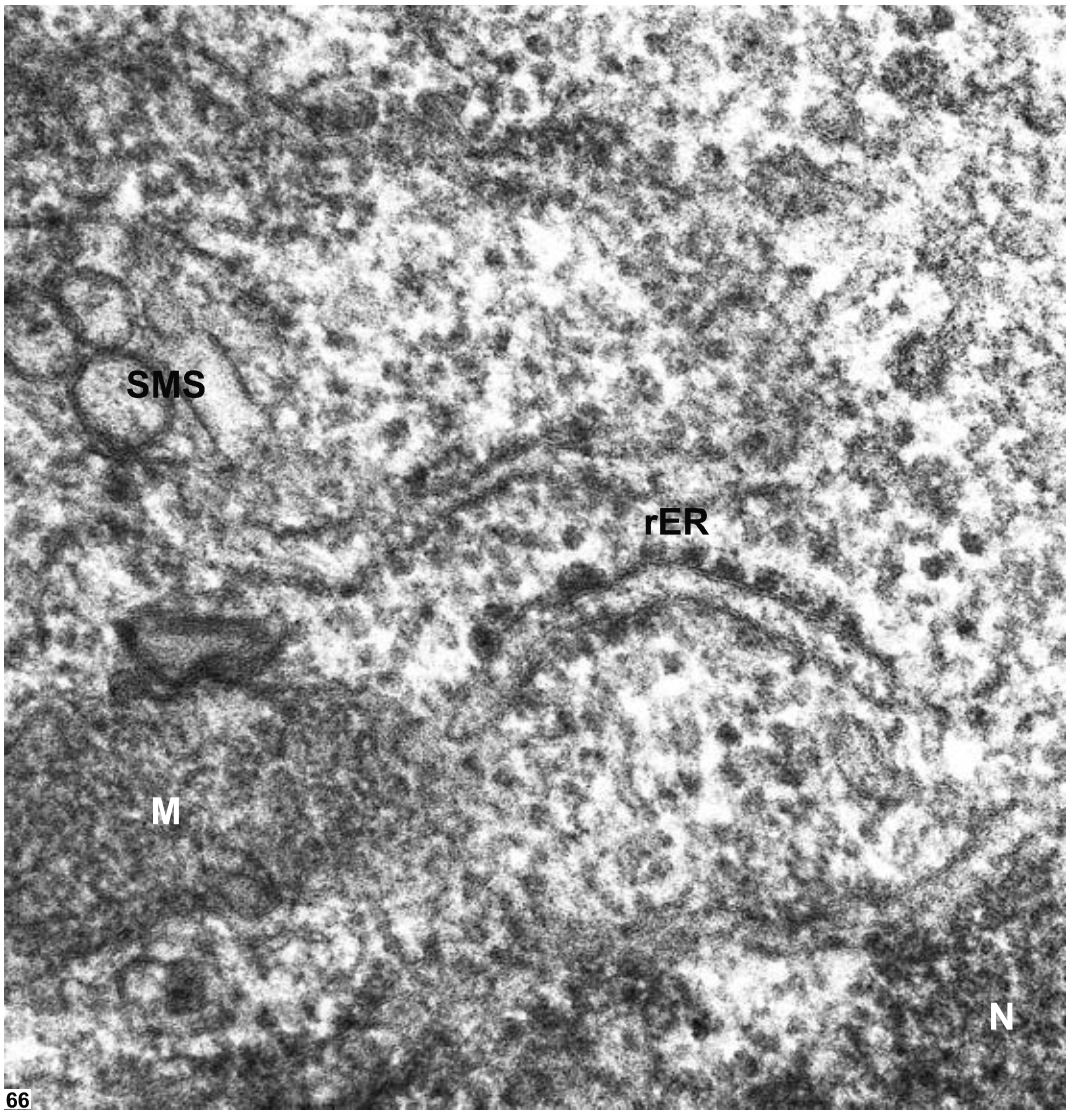


Fig. 66. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 6 days post-infection and standard processed as described in Fig. 40.

Original magnification: 50,000x. Final magnification: 137,000x.

The cystern of the rough endoplasmic reticulum in the center of the figure presents ribosomes of increased size and electron density, signalizing that viral morphogenesis is in progress. Above, at the left side of the figure, vesicles and one tubule of smooth membrane structures occur inside a cystern of the rough endoplasmic reticulum; they are limited by an unit membrane and enclose a light-gray stained material.

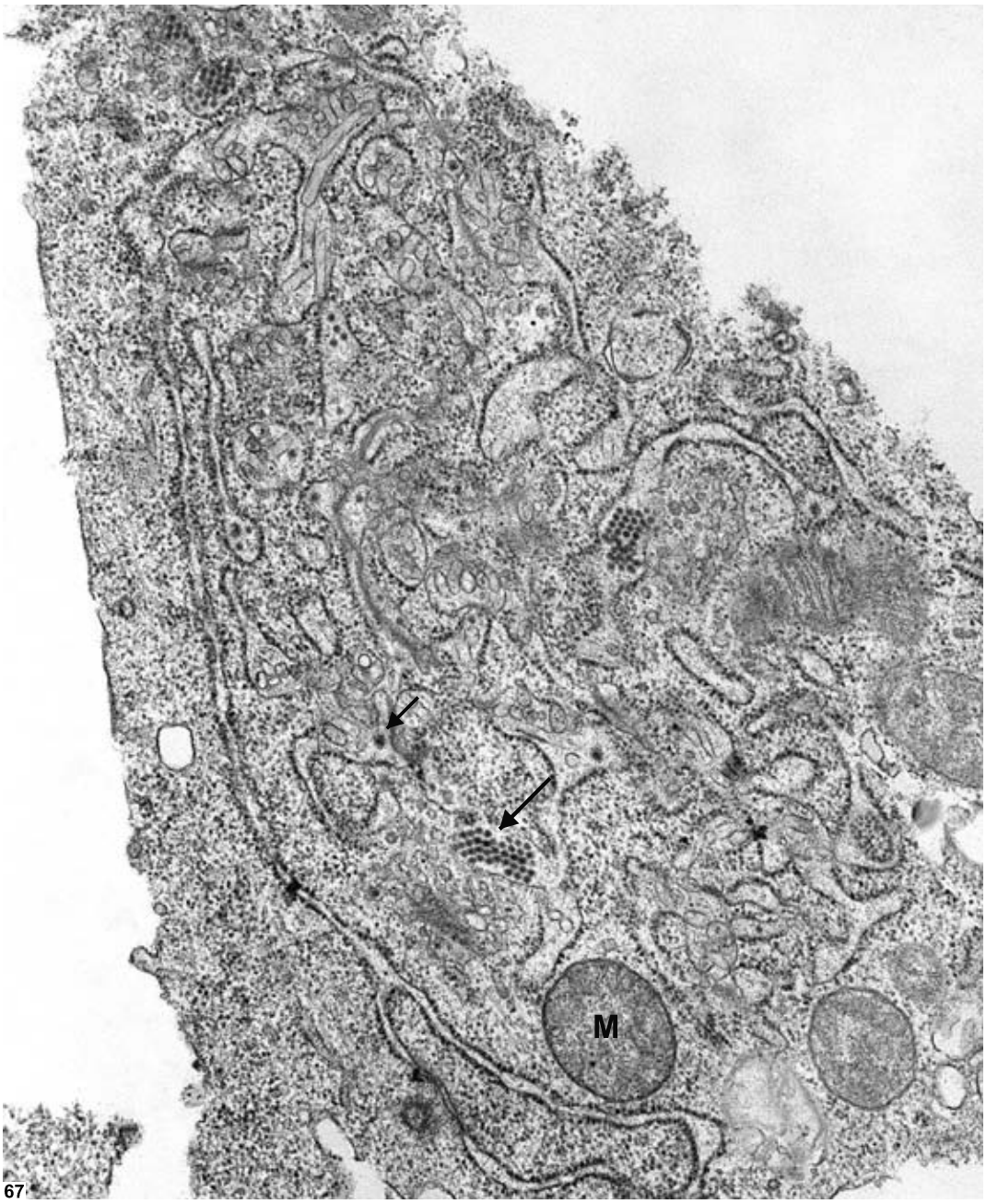


Fig. 67. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40. EDTA treatment of this section was carried out as described in Fig. 62.

Original magnification: 12,000x. Final magnification: 32,000x.

The area inside a syncytium where virus particle morphogenesis is in progress, is characterized by a well developed and distended rough endoplasmic reticulum, which cysterns contain the following structures: typical structured virus particles (long arrow), fuzzy coated virus-like particles (small arrow) and smooth membrane structures. No one of these structures was discolored by EDTA-treatment.

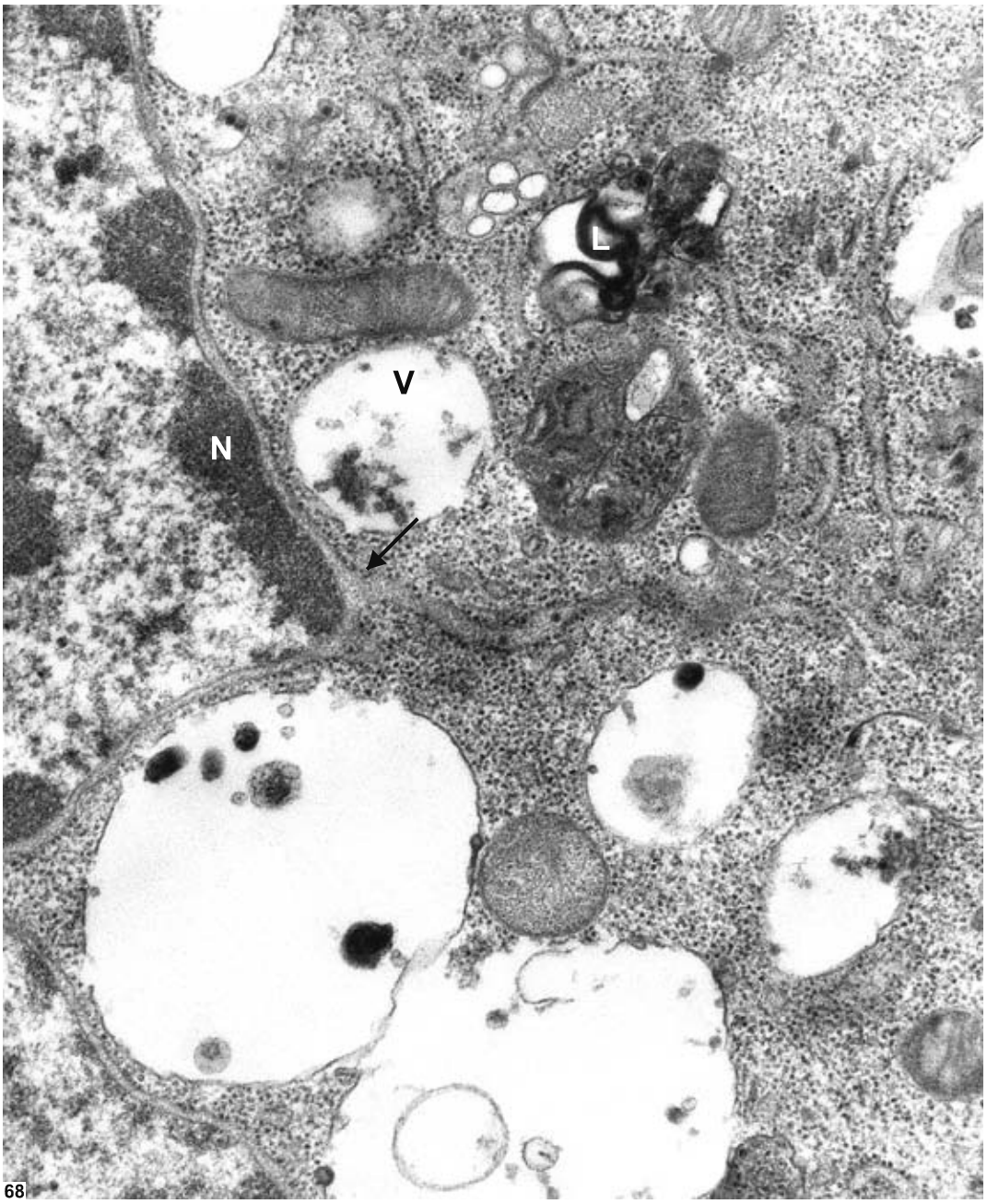


Fig. 68. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 16,500x. Final magnification: 43,000x.

The nuclear envelope and a cystern of the rough endoplasmic reticulum containing virus particles are connected (arrow). The presence of large vacuoles and lysosomes shows that cell infection is in an advanced stage of progression. Several cysterns of the rough endoplasmic reticulum contain virus particles (dark dots) and smooth membrane structures (light vesicles) in the upper central part of the figure.

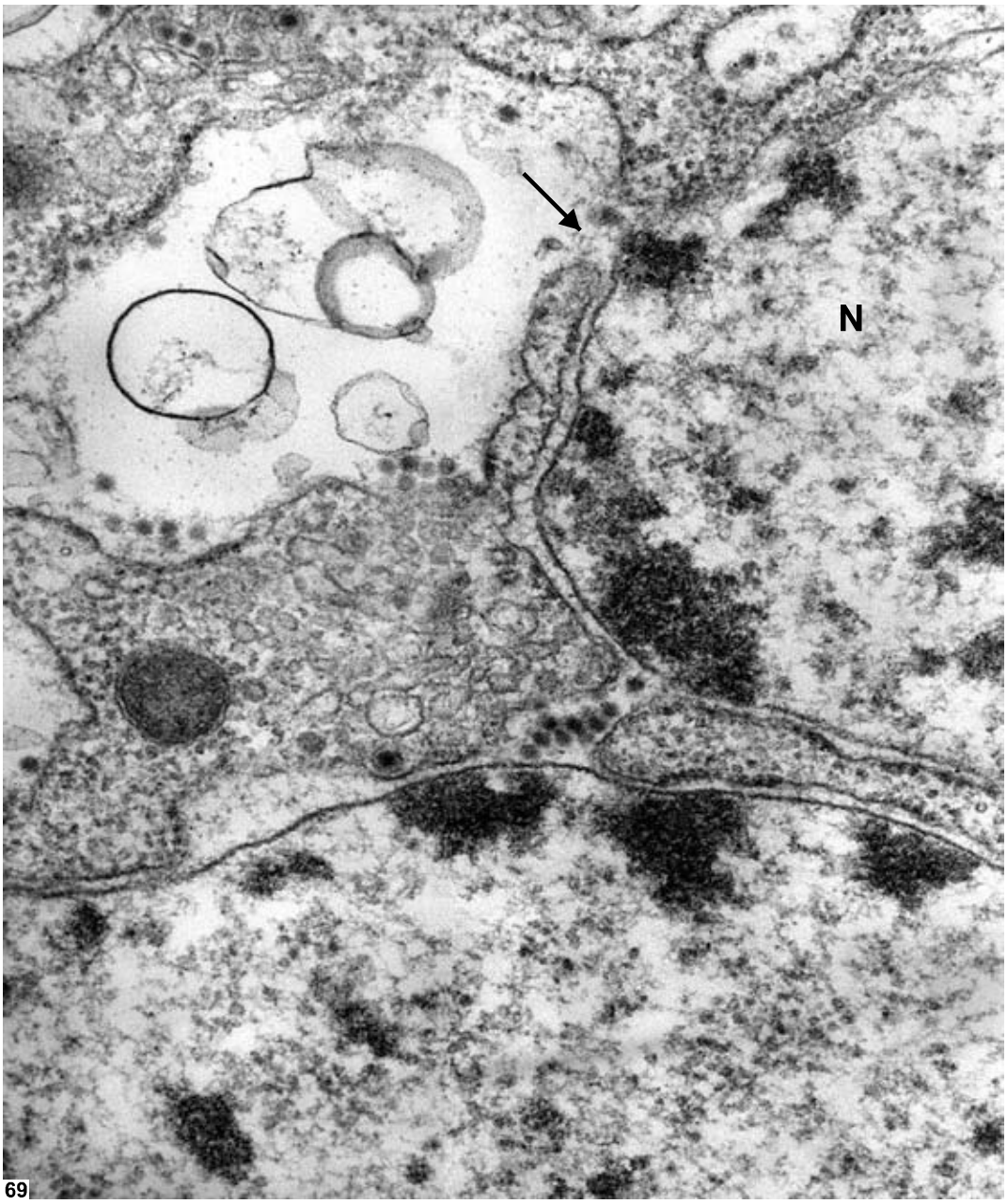


Fig. 69. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 66.

Original magnification: 20,000x. Final magnification: 55,000x.

A cystern of the rough endoplasmic reticulum is fused with the envelopes of two nuclei like a bridge. It contains numerous virus particles. Another, very large cystern of the rough endoplasmic reticulum fused also with the nuclear envelope (arrow); it presents virus particles and different sized vesicles inside, signalizing that virus morphogenesis is in progress. Cysterns and vesicles of a Golgy complex, one of them containing a dark stained virus particle, are in the center and probably also in the upper left corner of the figure.

Replication

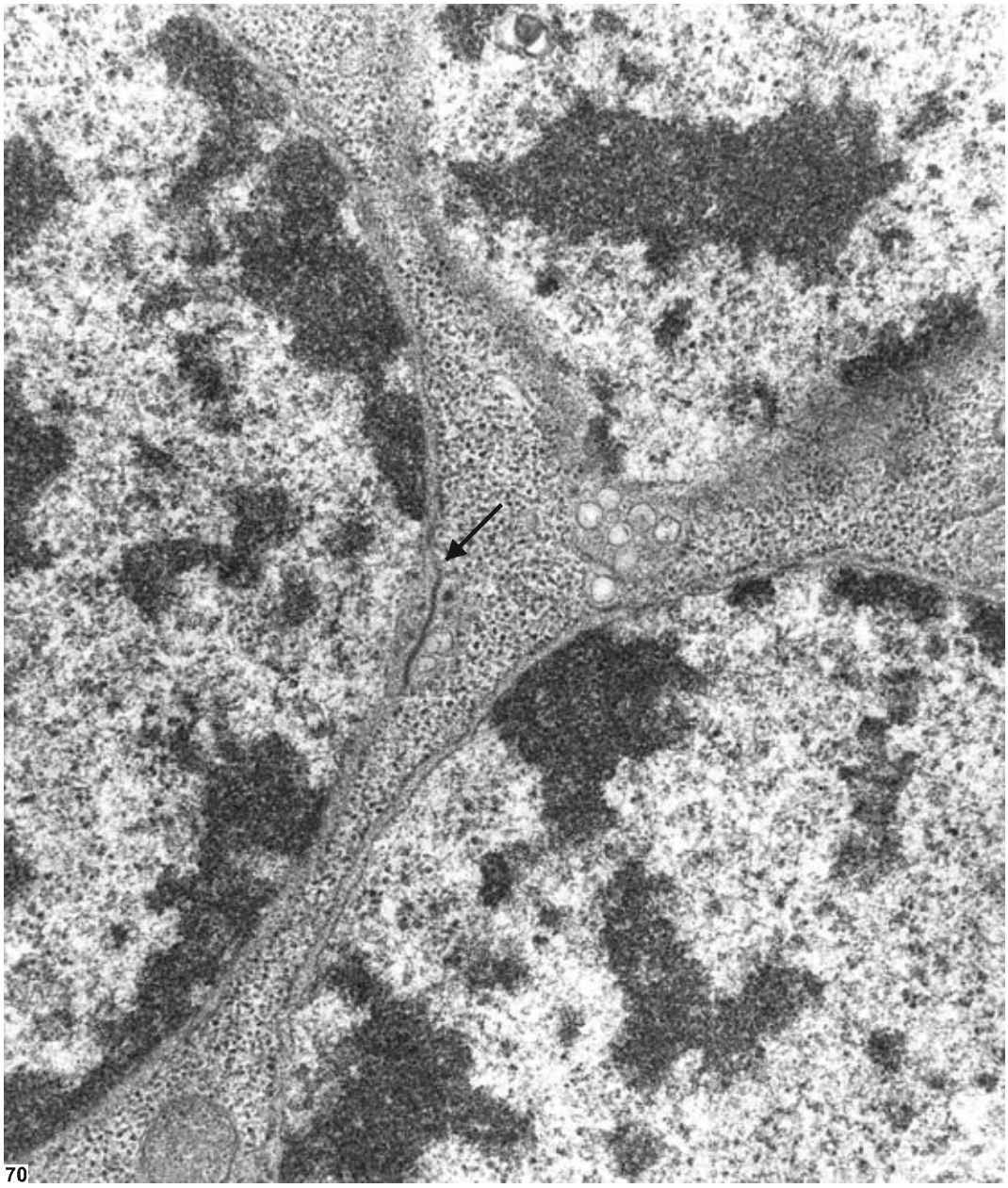


Fig. 70. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 16,500x. Final magnification: 45,000x.

A virus particle and smooth membrane structures containing cystern of the rough endoplasmic reticulum (arrow), is adjacent to the also virus particles containing nuclear envelope. Note the dark stained granular material between the two cysterns. Another cystern of the rough endoplasmic reticulum, containing a group of smooth membrane structures, is located also between two nuclei of the syncytium.

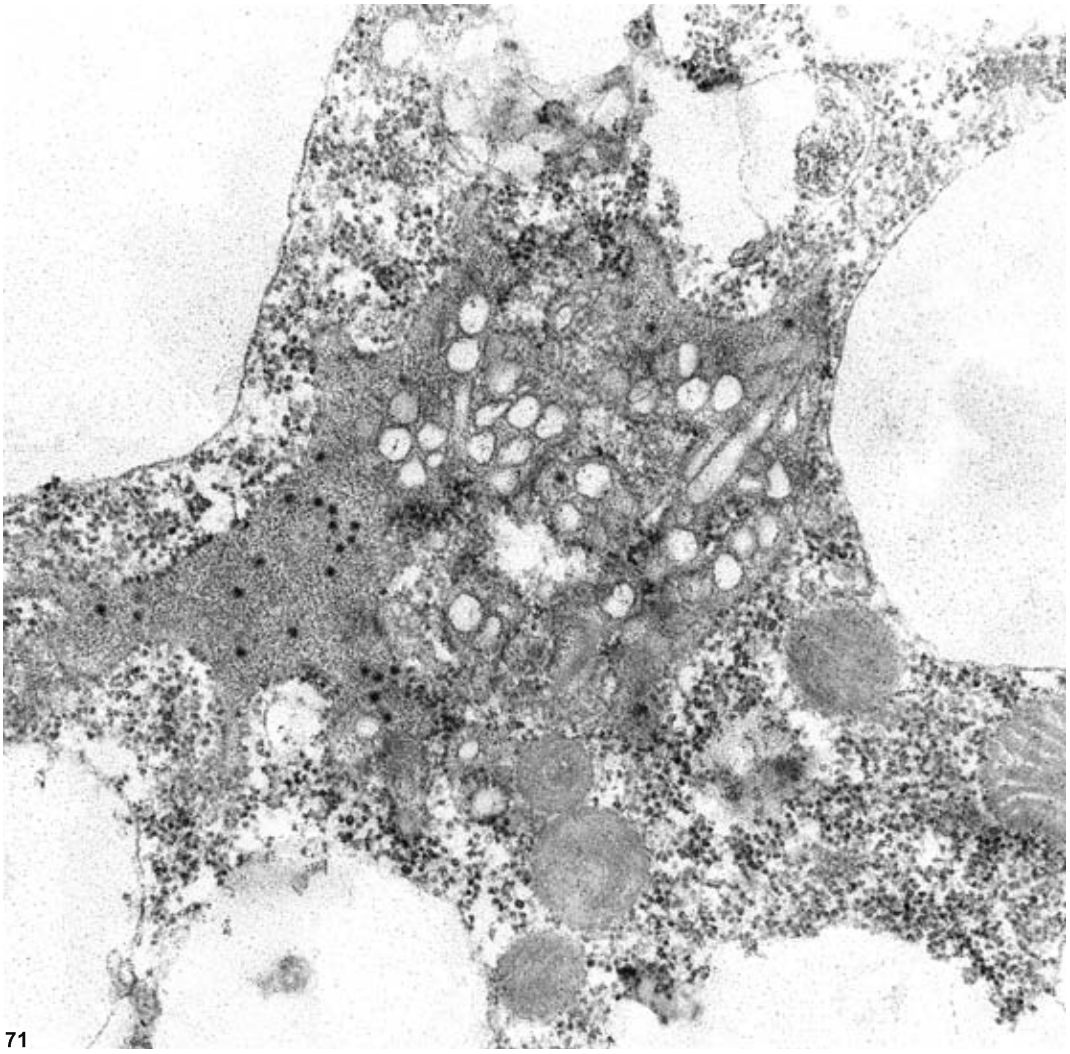


Fig. 71. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 16,500x. Final magnification: 43,000x.

At an advanced stage of syncytium development, a large cystern of the rough endoplasmic reticulum is fullfilled with a gray-stained homogeneous material of possible proteinous nature, dark-stained virus particles and light vesicles and tubules of smooth membrane structures. Vacuoles are randomly disposed. EDTA-treatment has had no effect on these structures.

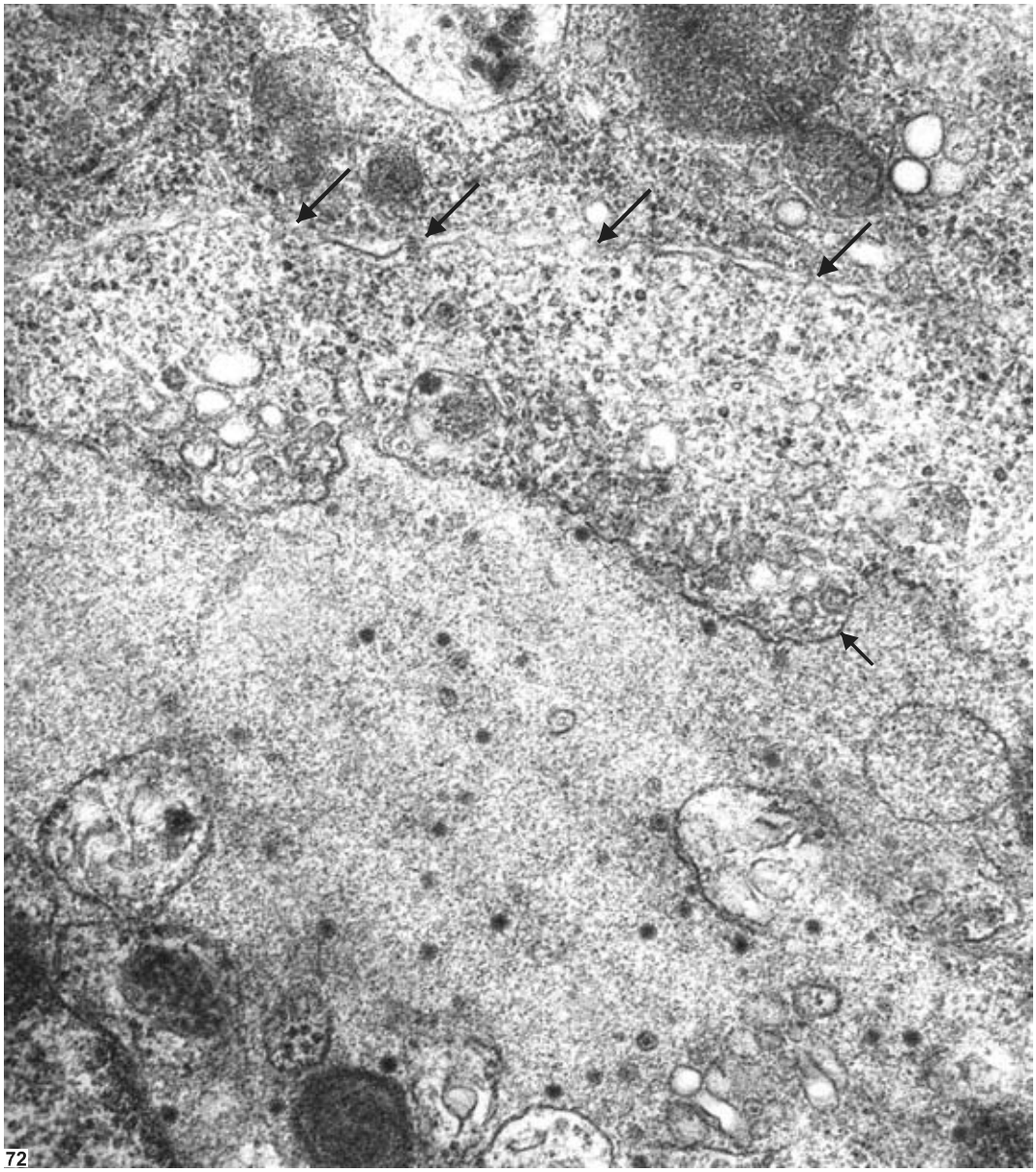


Fig. 72. Dengue-1 virus infected mosquito cell culture. TEM

The cell culture was fixed 8 days post-infection and standard processed as described in Fig. 40.

Original magnification: 20,000x. Final magnification: 51,000x.

At an more advanced stage of virus replication and morphogenesis, a large cystern of the rough endoplasmic reticulum lost the ribosomes, mantaining only the unit membrane (small arrow). This large vesicle contains a homogenous gray stained possibly proteinous material, dark stained virus particles, smooth membrane structures and inclusion bodies. Several connections (large arrows) between the two virus infected cells may be detected in the upper side of the figure (compare also with Figs. 30 and 57).

Replication

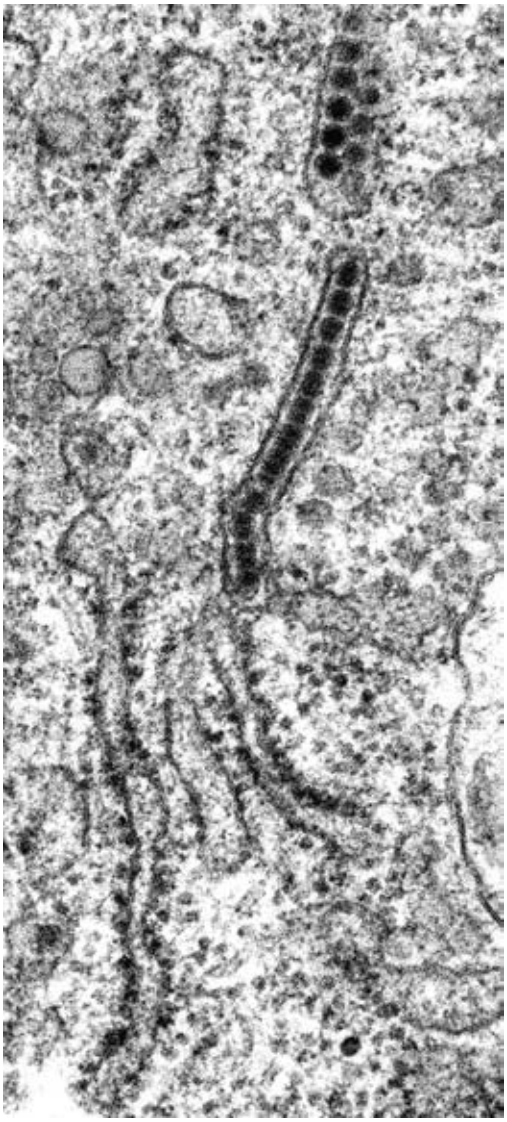


Fig. 73. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 30,000x. Final magnification: 79,000x.

Viral nucleocapsids and envelopes are formed inside the cysterns of the rough endoplasmic reticulum. Swollen ribosomes are connected to the cytoplamic face of the rER-unit membrane of an endoplasmic reticulum cystern at the lower left side of the figure. At the center and upper side of the figure, viral particles are aligned inside cysterns now lacking ribosomes.

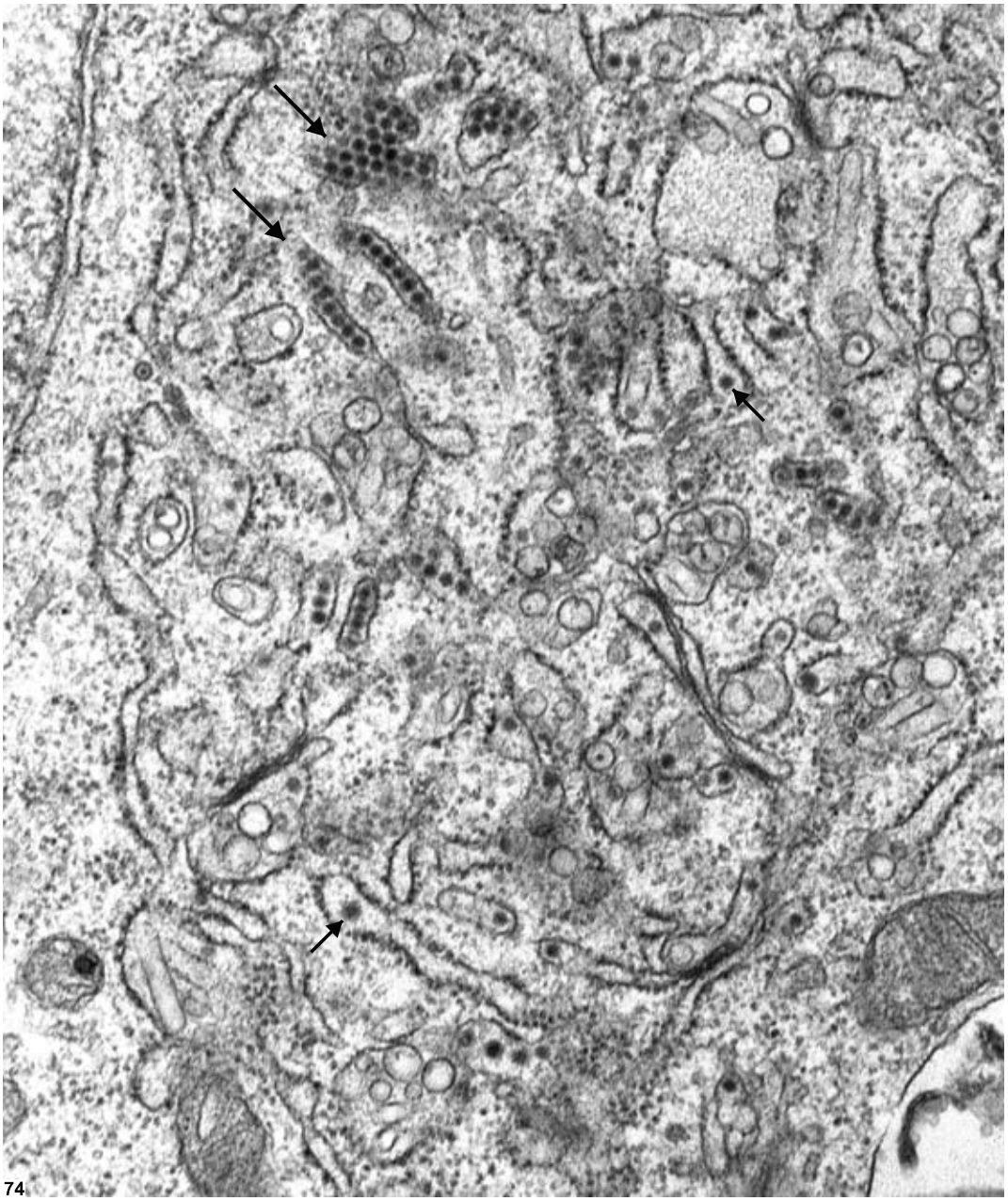


Fig. 74. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 67.

Original magnification: 20,000x. Final magnification: 46,000x.

At a more advanced stage of viral morphogenesis, distended cysterns of the rough endoplamic reticulum include groups of aligned virus particles of typical morphology (long arrows), fuzzy coated atypical virus-like particles (small arrow) and smooth membrane structures. Ribosomes are sometimes absent of the rough endoplasmic reticulum membranes.

Replication

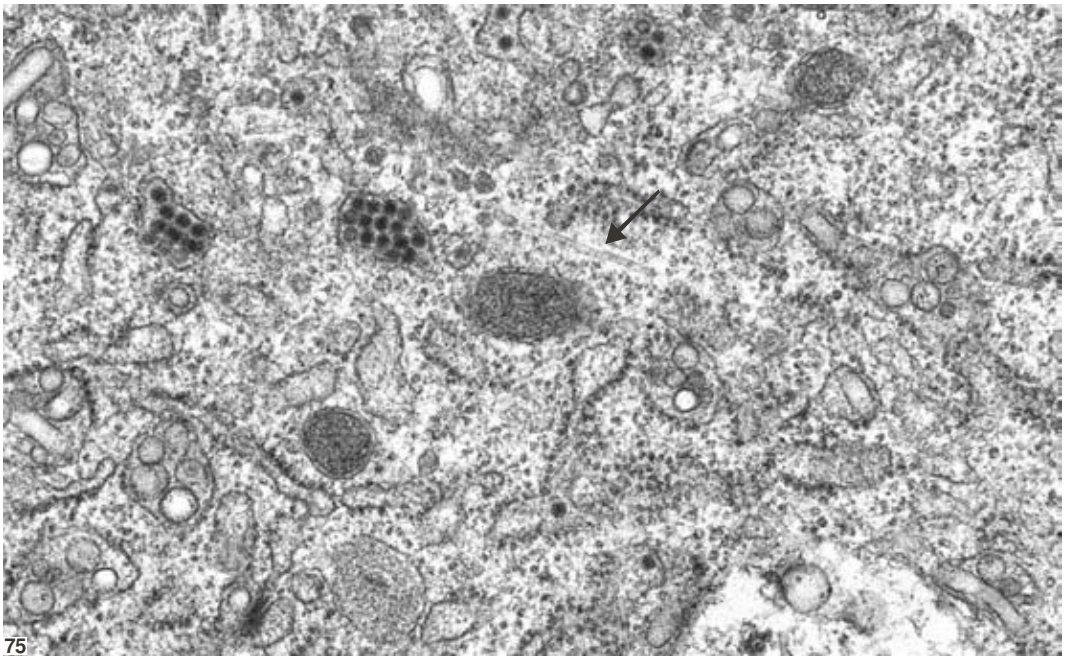


Fig. 75. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 73.

Original magnification: 20,000x. Final magnification: 49,000x.

Typical structured virus particles are found single or assembled inside cysterns of the rough endoplasmic reticulum, devoid of ribosomes as the infection is in progress. A microtubule (arrow) can be observed also in the cell region of virus morphogenesis.

Replication

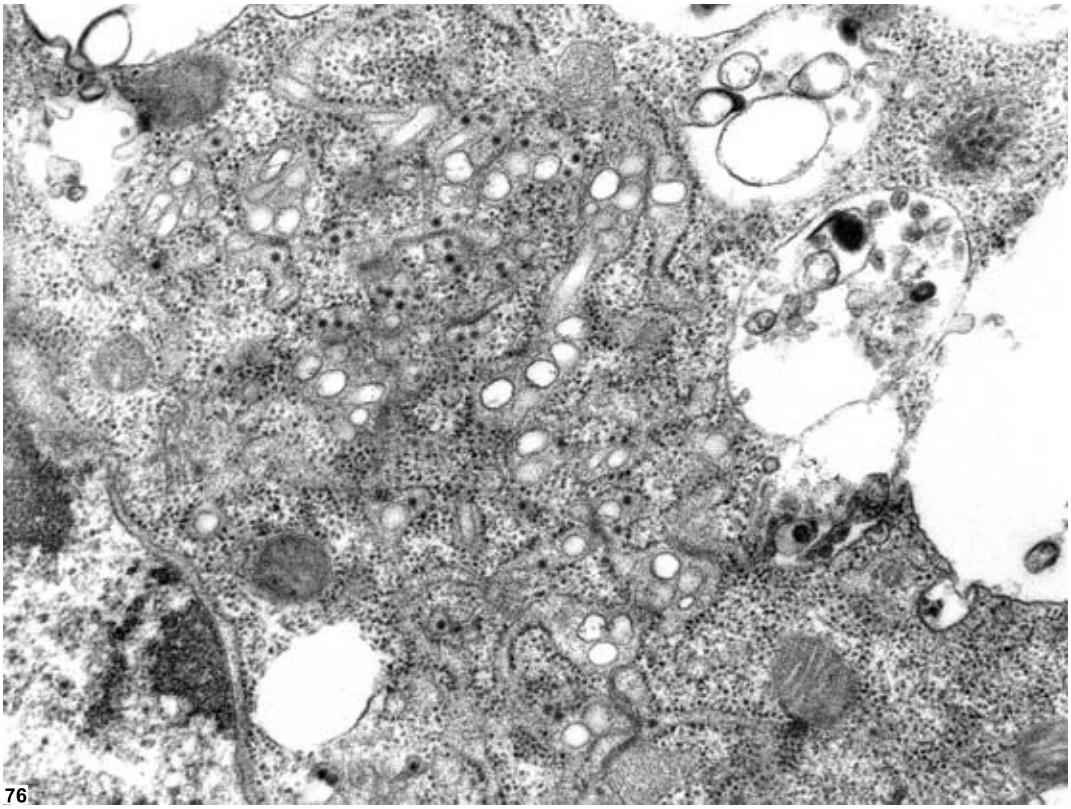


Fig. 76. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 16,500x. Final magnification: 38,000x.

Virus particles and smooth membrane structures are frequent inside cysterns of the rough endoplasmic reticulum, partially devoid of ribosomes. Smooth membrane structures occur as vesicles and tubules; some of them are electron-translucent and may have lost its content during cell processing; other ones present a gray stained content and irregularly disposed filaments inside, as it is better visualized in Fig. 77.

Replication

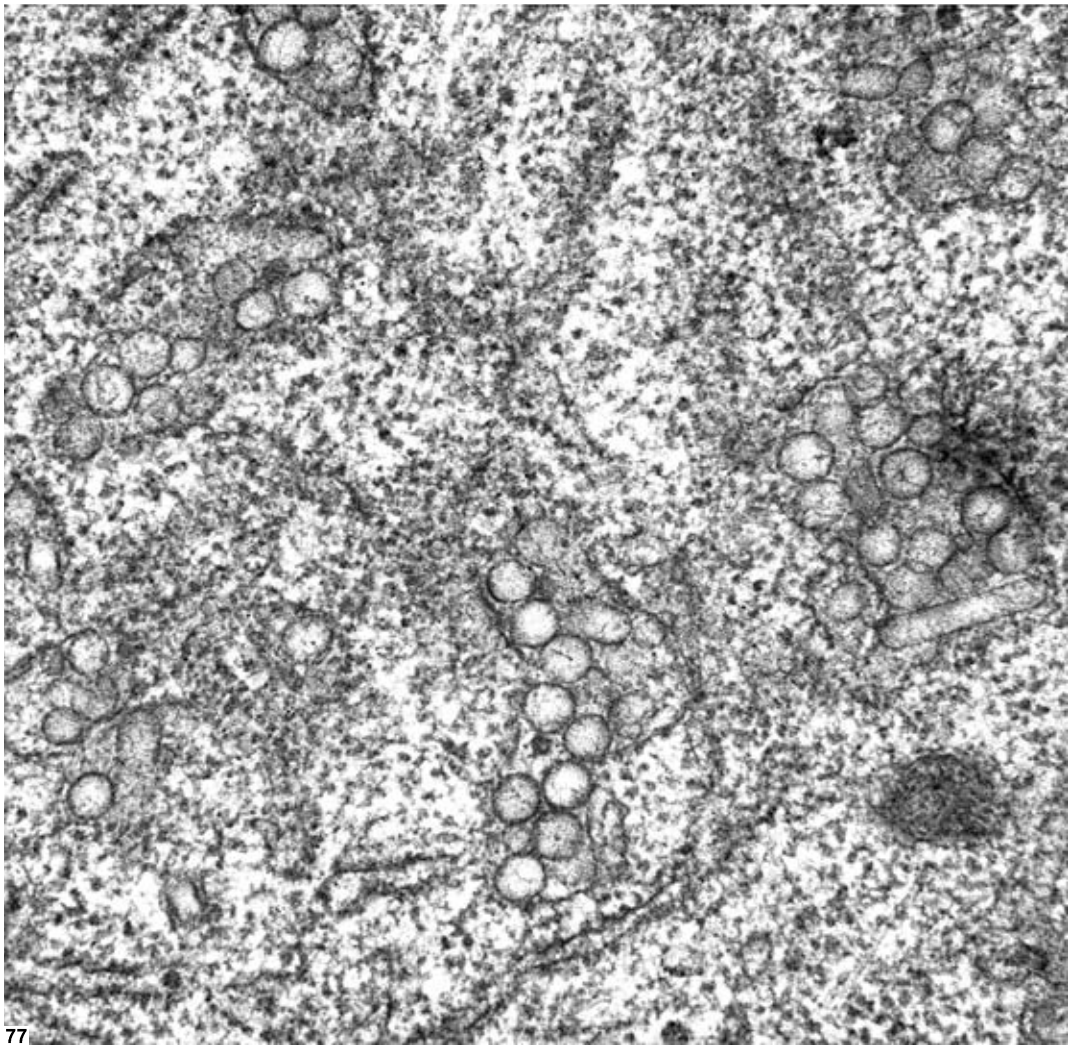


Fig. 77. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 30,000x. Final magnification: 68,000x.

The good preserved smooth membrane structures (numerous vesicles and one tubule) present a homogeneous content and fine electron-dense filaments. They are grouped in packets inside cysterns of the rough endoplasmic reticulum.

Replication

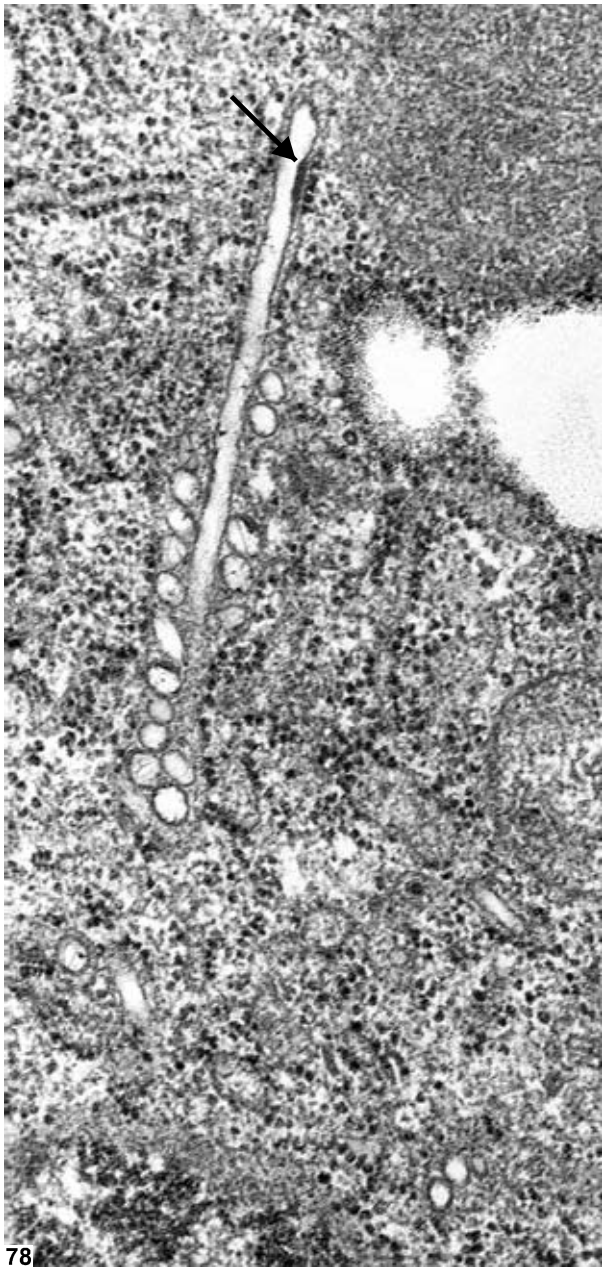


Fig. 78. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture of the 4th virus passage was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 20,000x. Final magnification: 49,000x.

. One cystern of the rough endoplasmic reticulum contains a very long tubule and several vesicles of smooth membrane structures. Ribosomes are swollen at the upper right outer side of the cystern and dark stained material of probably RNA-nature is located over the tubule membrane (arrow), inside the cystern.

Replication

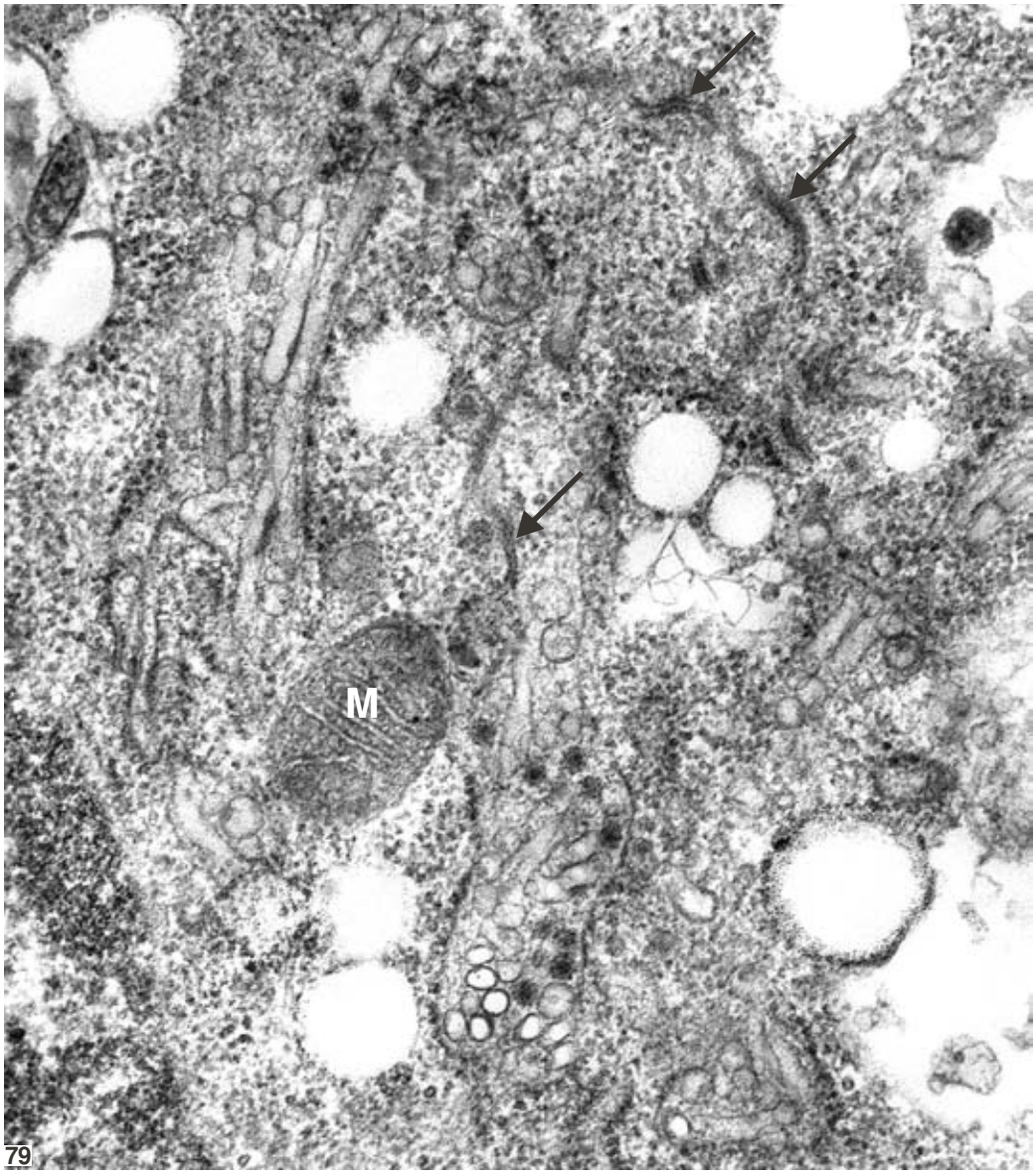


Fig. 79. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 78.

Original magnification: 20,000x. Final magnification: 49,000x.

The figure presents a later stage of viral replication. Smooth membrane structures inside cysterns of the rough endoplasmic reticulum, partially lacking attached ribosomes, are numerous; tubular forms are more frequent. Atypical, fuzzy coated virus-like particles are numerous inside the central cystern of the figure. Lysosomes and electron-translucent large vesicles that probably lost their contents during cell culture processing for sections, are very common. During this stage of cell degeneration double-track-structures (arrows) appear when two cysterns of the rough endoplasmic reticulum are adjacent; these structures are similar to confrontant cysterns as described for cells of several tissues (Ghadially, 1982). They are not discolored by EDTA-treatment and may contain some RNA.

Replication

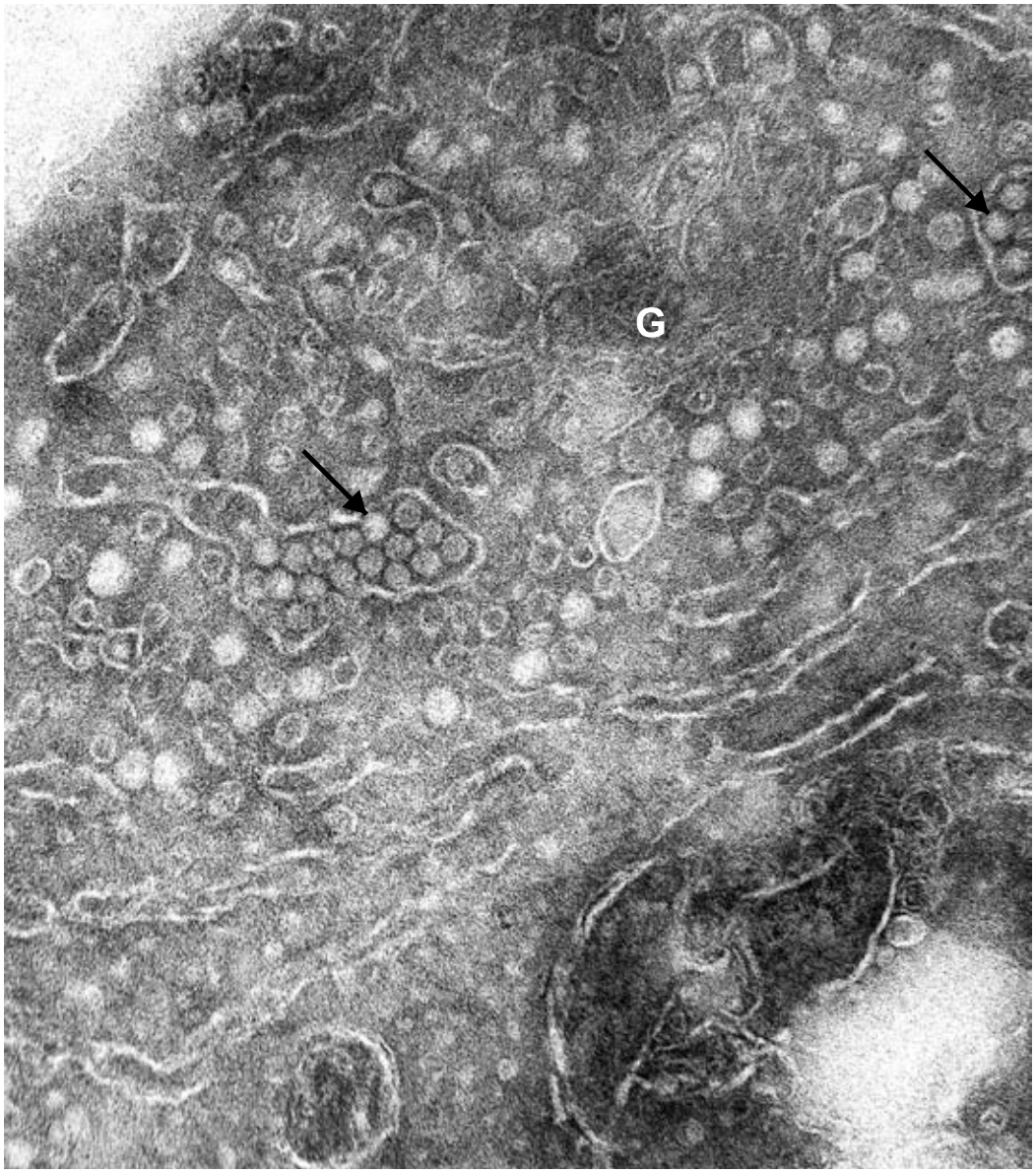


Fig. 80. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection in 1% phosphate buffered glutaraldehyde. Ultrathin sections were obtained using the crioultramicrotomy technique (Griffith, 1993), in order to obtain more information about localization of virus particle and smooth membrane structures inside cell organelles.

Original magnification: 30,000x. Final magnification: 74,000x.

This typical image of a cryosection shows virus particles densely assembled in vesicles (arrows), the rough endoplasmic reticulum which ribosomes are not distinguished by this technique, cysterns and vesicles of the Golgi complex. Smooth membrane structures are difficult to be identified by this technique, because they have maintained its content during processing.

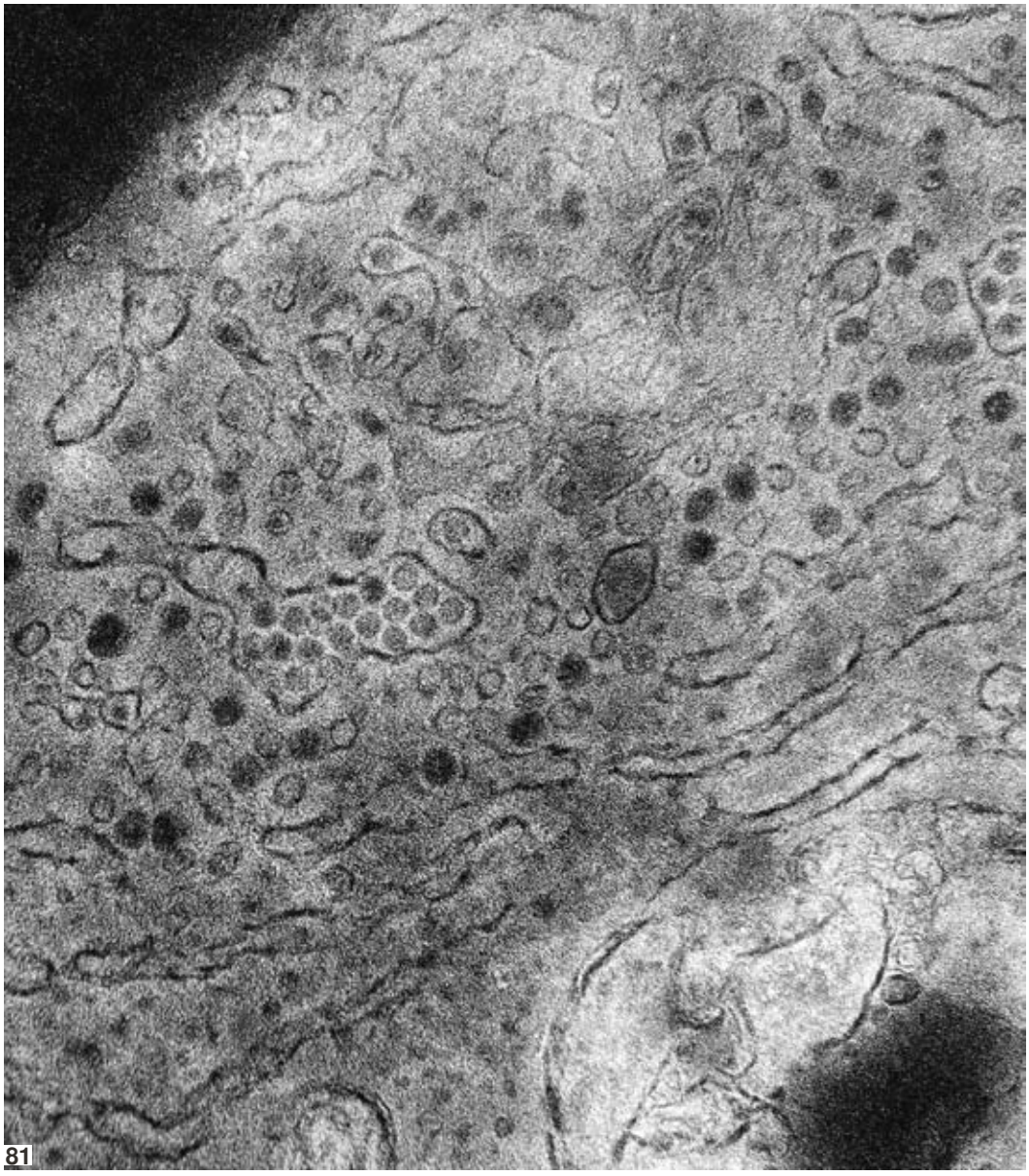


Fig. 81. Dengue-2 virus infected mosquito cell culture. TEM

The same image as in Fig. 80 using the computer “invert” option. Unit membranes and virus nucleocapsids now are dark-gray stained, while “empty” vesicles are lighter. This image is more similar to the Epon embedded samples.

Replication

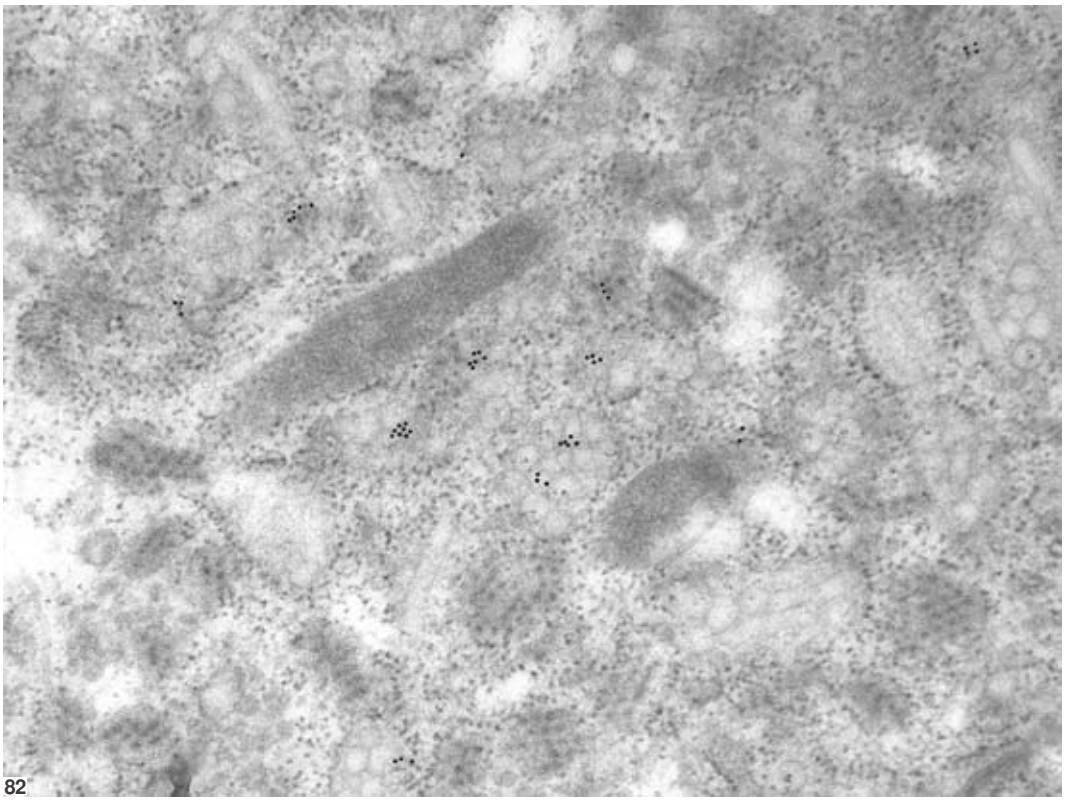


Fig. 82. Dengue-2 virus infected mosquito cell culture. TEM-ISH

The infected cell culture was fixed using cacodylate buffered 1% glutaraldehyde, dehydrated using increasing concentrations of ethanol and low temperature embedded in LR-Gold resin. Ultrathin sections were harvested on nickel grids, incubated using digoxigenin labelled probe and stained with aqueous uranyl acetate only (Barth, 1999; Grief et al., 1997).

Original magnification: 22,000x. Final magnification: 62,000x.

In situ hybridization of dengue-2 virus shows that single stranded viral RNA (ssRNA) can be detected on the ribosomes of the rough endoplasmic reticulum and on smooth membrane structures. Enveloped virus particles and viral nucleocapsids never present the hybridization signal.

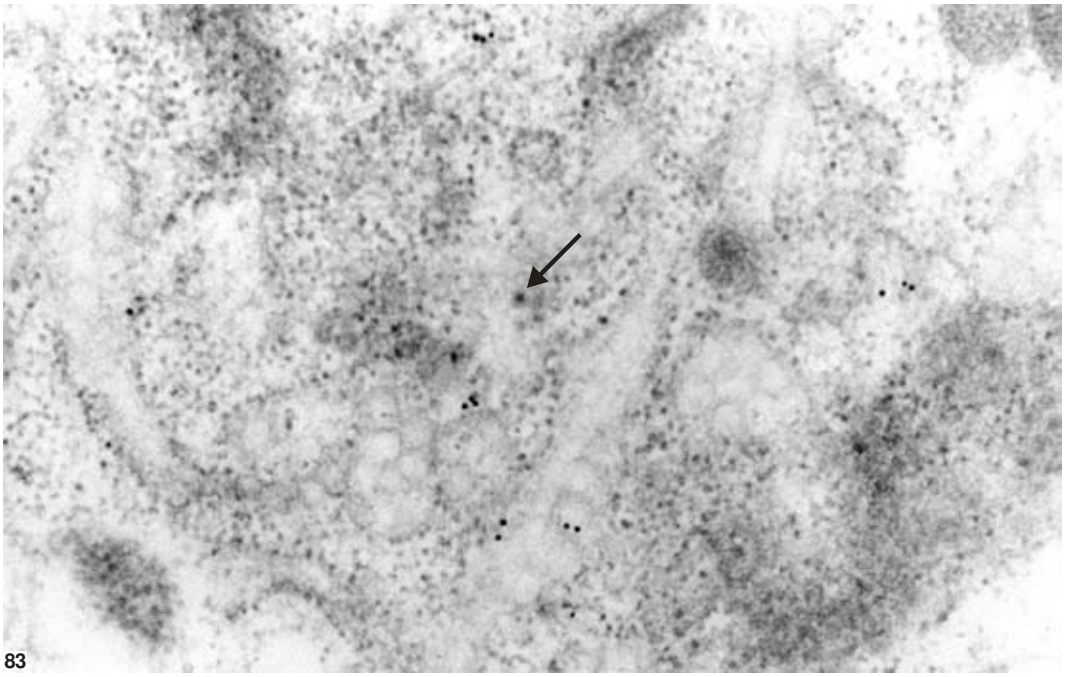


Fig. 83. Dengue-2 virus infected mosquito cell culture. TEM-ISH

The same preparation as in Fig. 82
Original magnification: 22,000x. Final magnification: 75,000x.
The hybridization signal is found on the smooth membrane structures. An enveloped virus particle shows no signal (arrow).

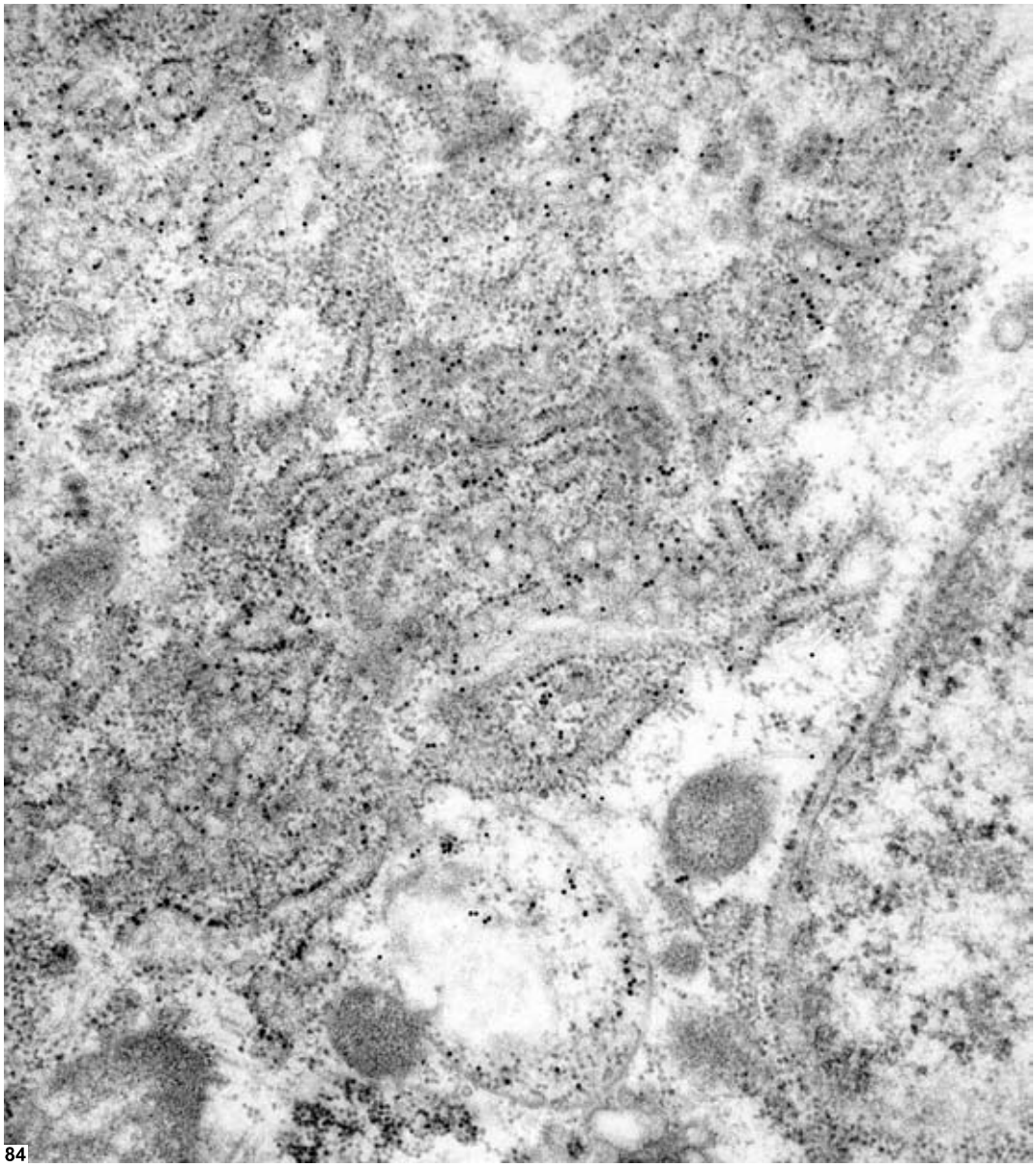


Fig. 84. Dengue-2 virus infected mosquito cell culture. TEM-IEM

The same preparation as in Fig. 82.

Original magnification: 20,000x. Final magnification: 46,000x.

Ultrathin sections were harvested on nickel grids, formerly incubated using a hiperimmune ascitic fluid and in sequence incubated with 10nm gold-protein A and stained with aqueous uranyl acetate and lead citrate.

Original magnification: 20,000x. Final magnification: 50,000x.

Antibody linked gold particles are mostly found on smooth membrane structures and rough endoplasmic reticulum membranes. Cell nucleus and mitochondria are not labelled. Cytosol, microtubules and nucleus at the right side of the figure are also not labelled.

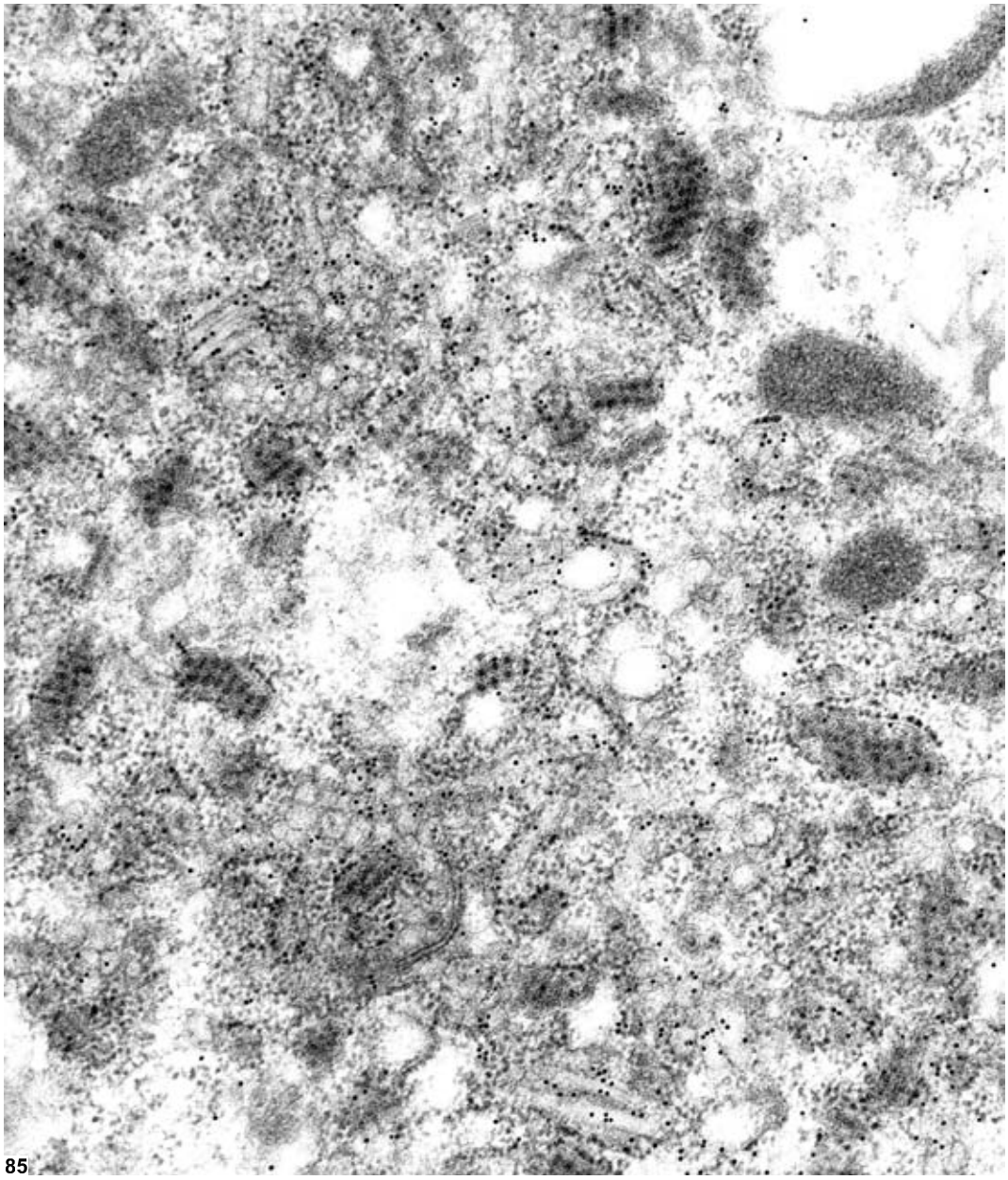


Fig. 85. Dengue-2 virus infected mosquito cell culture. TEM-IEM

The same preparation as in Fig. 82.
Original magnification: 20,000x. Final magnification: 50,000x.

Packets of virus particles show no label, while smooth membrane structures show surface labelling. Mitochondria are not labelled.

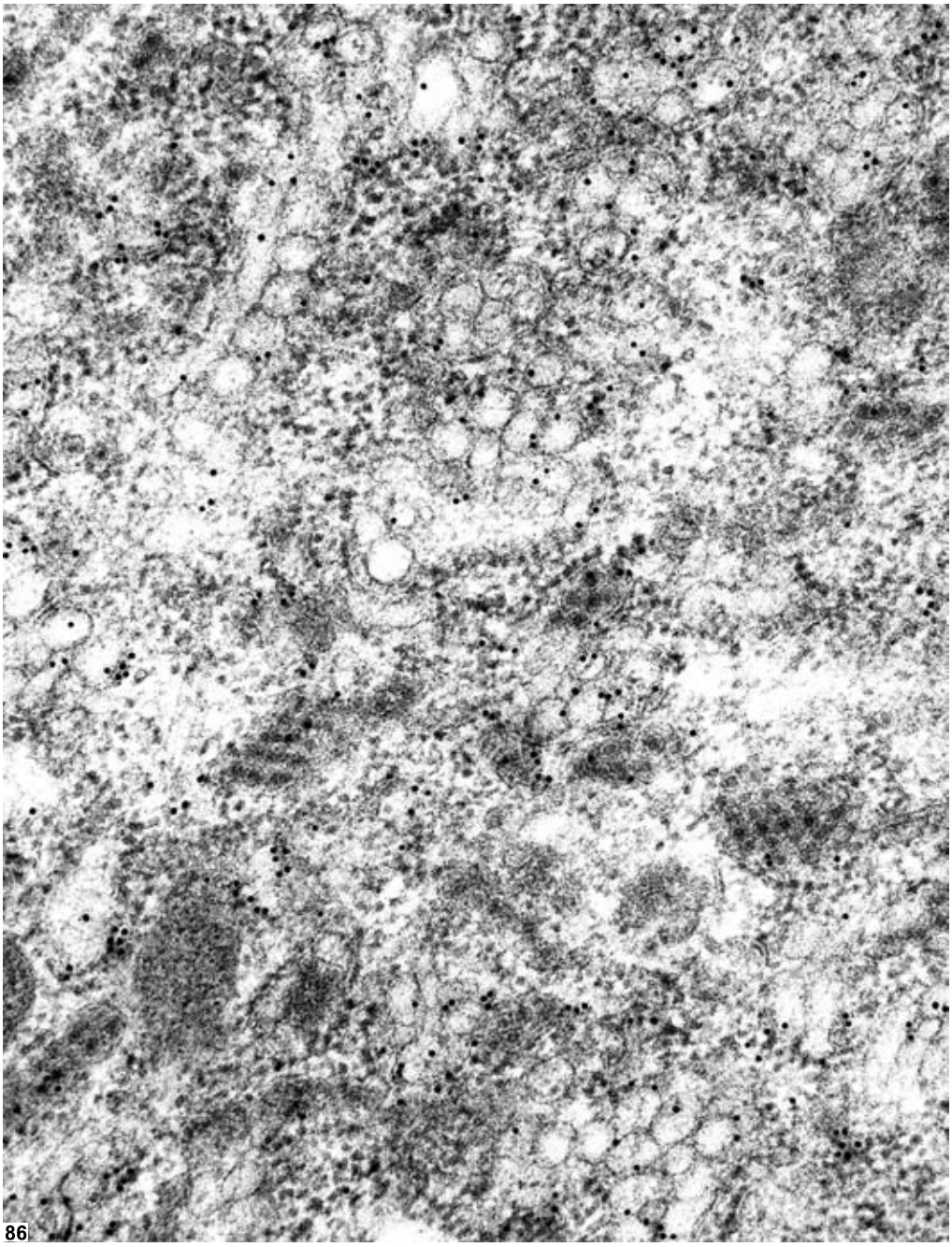


Fig. 86. Dengue-2 virus infected mosquito cell culture. TEM-IEM

The same preparation as in Fig. 82.

Original magnification: 30,000x. Final magnification: 75,000x.

Packets of virus particles and smooth membrane structures (vesicles and tubules) are inside enlarged cysterns of the rough endoplasmic reticulum. Gold label is found mostly over the smooth membrane structures. Viral nucleocapsids are rarely labelled, while ribosomes and mitochondria are not labelled at all.

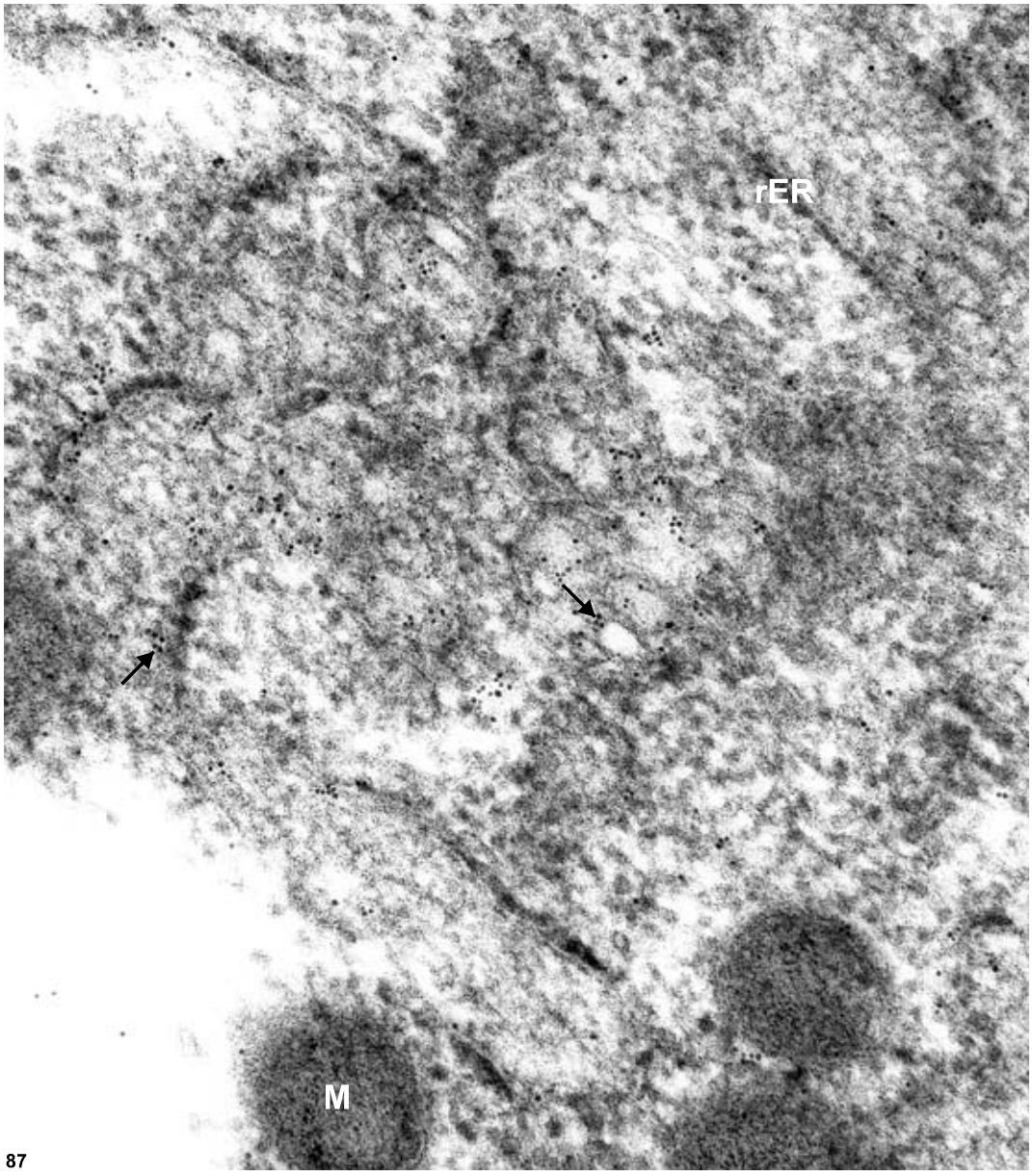


Fig. 87. Dengue-2 virus infected mosquito cell culture. TEM-IEM

The same preparation as in Fig. 82.

Original magnification: 50,000x. Final magnification: 130,000x.

This tangential section of smooth membrane structure membranes shows that gold label is located over the membranes and not inside the vesicles (arrows). Cytosol and mitochondria show no gold label.

Replication

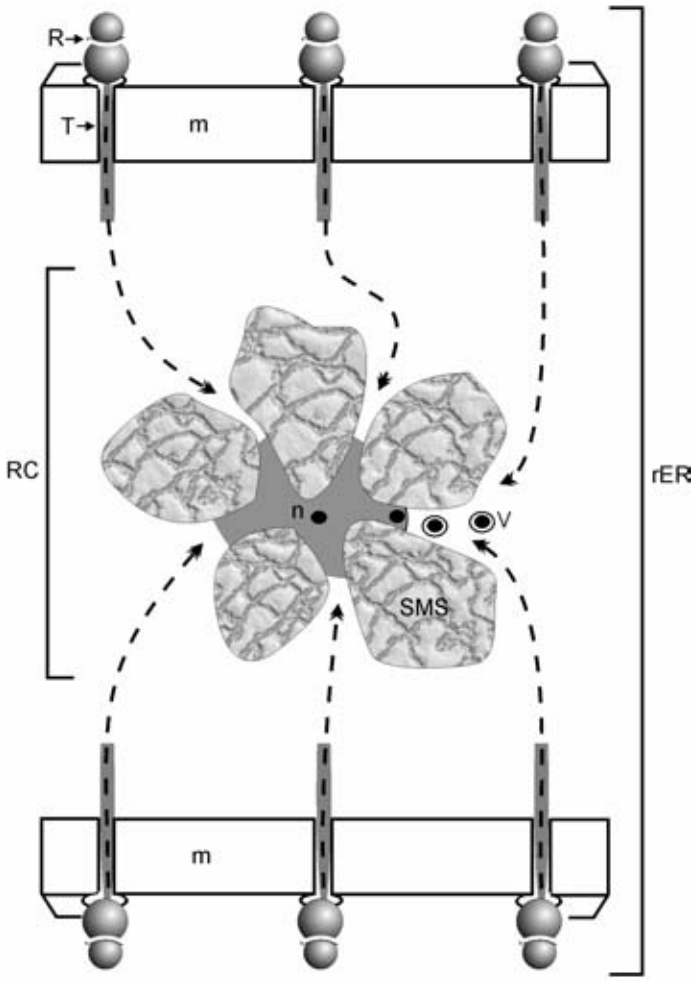


Fig. 88. A model for enveloped dengue virus particle morphogenesis.

Hypothetical way of virus particle synthesis. Viral RNA can pass the translocator protein below the rER-linked ribosomes. Proteins are there synthetized into the rER lumen. Both migrate and anchore on the SMS surfaces and condense in the middle zone into viral nucleocapsids (virus RNA+ core protein). Emerging from this dense structured area, the nucleocapsids adquire the envelopes (containing protein prM and protein E) and became immature virus particles.
m= membrane lipid bilayer; n= viral nucleocapside; R= rER-membrane associated ribosomes; RC= viral replication complex; rER= rough endoplasmic reticulum; SMS= smooth membrane structures; T= translocator protein; V= enveloped immature virus particle; ----= pathway of viral protein and possible nucleic acid transfer. (from Barth, 1999, modified)

Replication

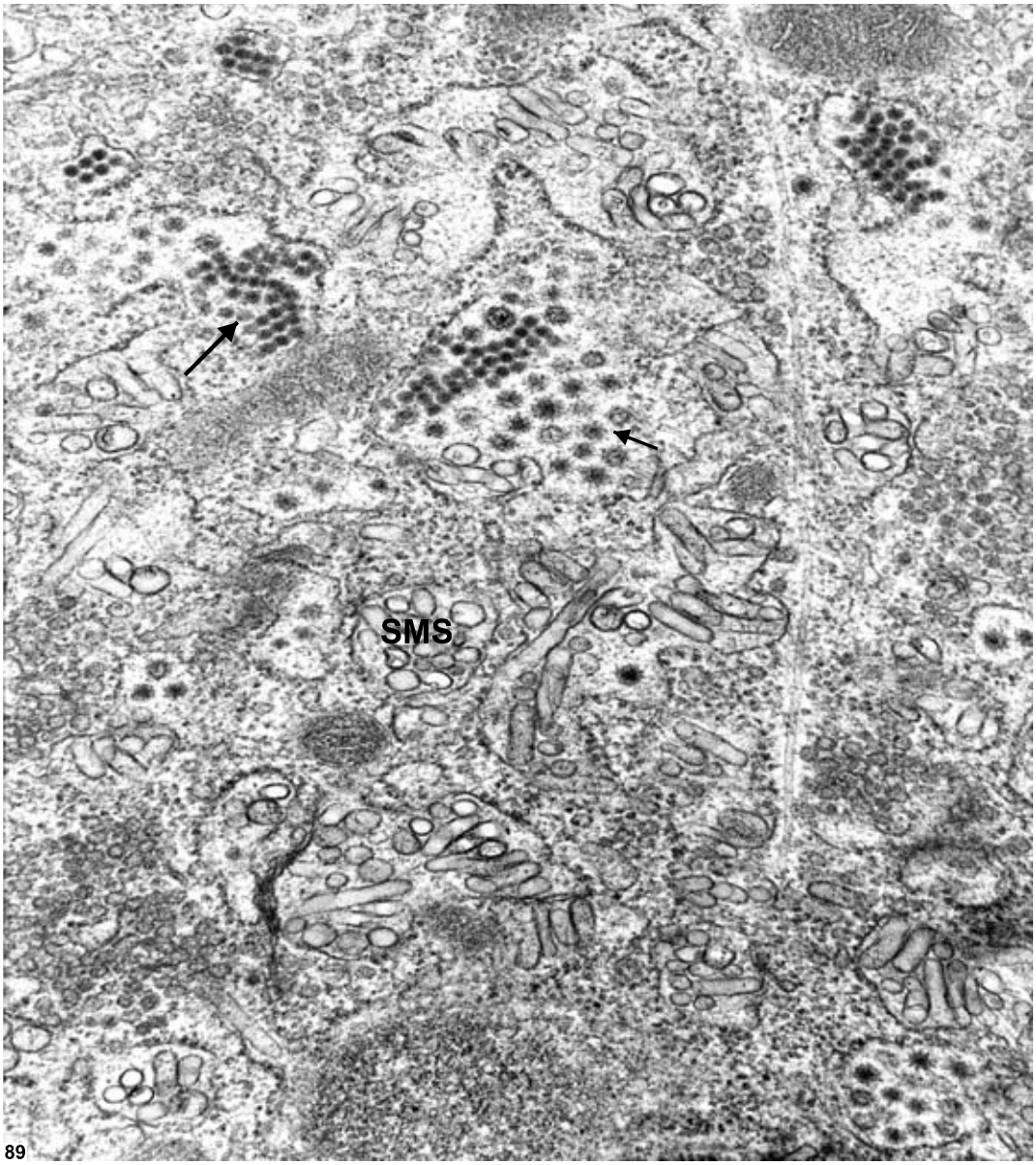


Fig. 89. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 66.

Original magnification: 20,000x. Final magnification: 46,000x.

Typical smooth (long arrow) and atypical virus-like fuzzy-coated (small arrow) virus particles, as well as tubules and vesicles of smooth membrane structures occur together inside the cysterns of the rough endoplasmic reticulum. Note a vertically placed microtubule at the right side of the figure.

Replication

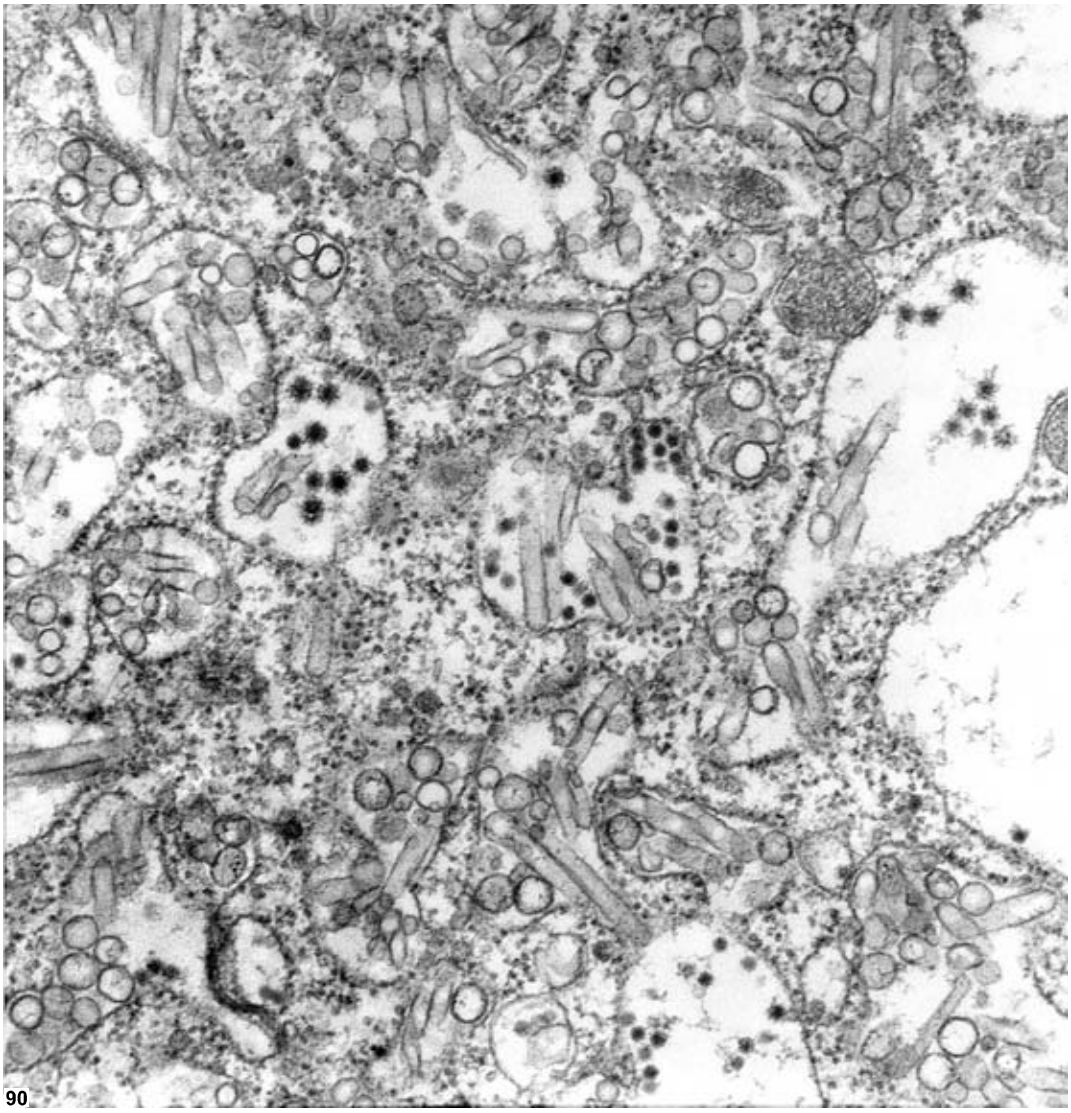


Fig. 90. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 20,000x. Final magnification: 47,000x.

Dengue-2 typical (smooth coated) and atypical (fuzzy coated) virus particles occur inside enlarged cysterns of the rough endoplasmic reticulum. Numerous membrane bound ribosomes of the rough endoplasmic reticulum are lost during viral morphogenesis progress.

The smooth membrane structures (which nature is yet unknown) appear sometimes electron translucent when its content was removed, probably during cell processing. Some of these structures show fine gray filaments and a homogeneous gray substance inside and may be of proteinous nature, indicating a better condition of preservation.

Replication

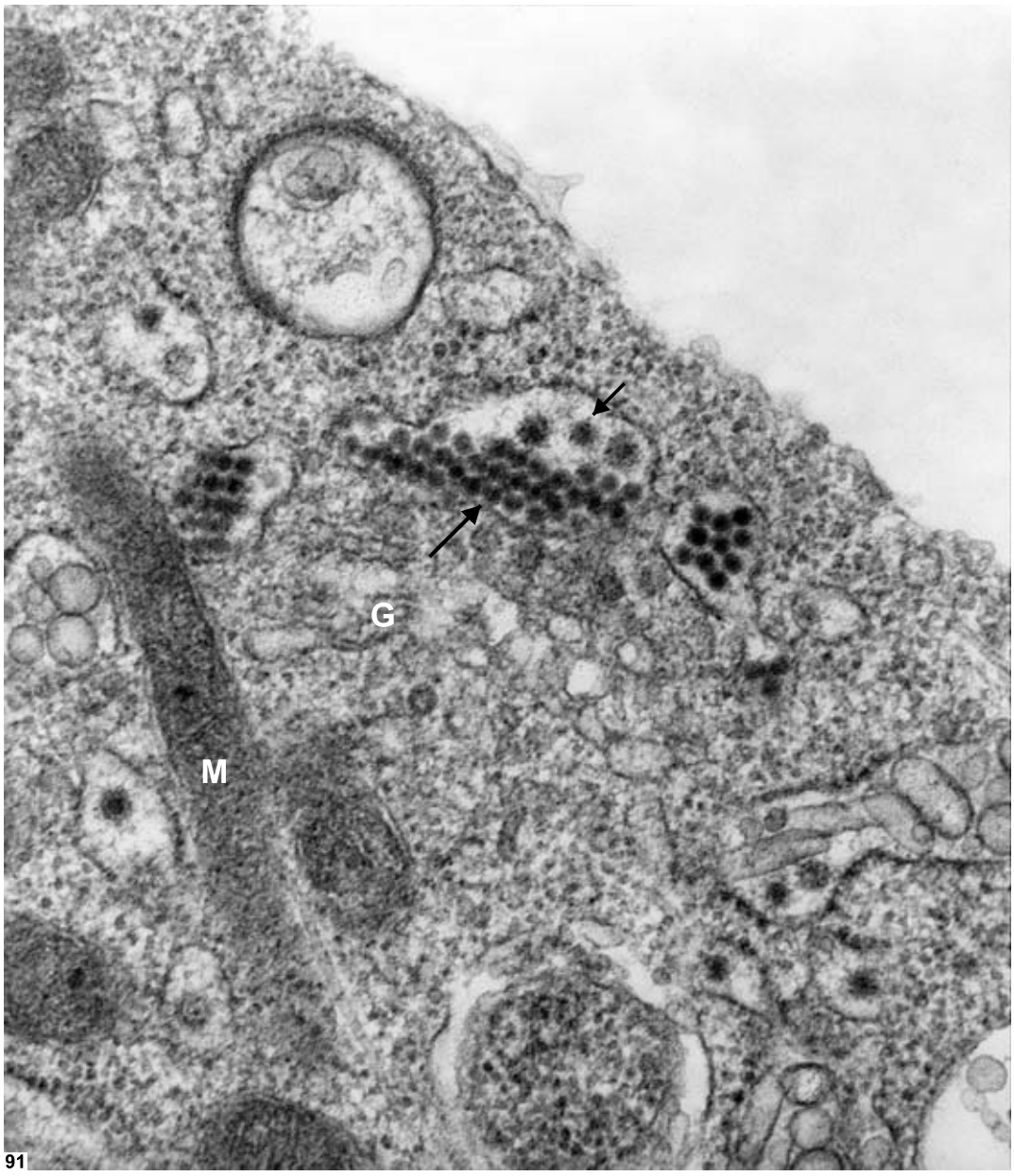


Fig. 91. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 66.

Original magnification: 30,000x. Final magnification: 68,000x.

Typical virus particles (long arrow) ordered in paracrystalline arrays occur simultaneously with atypical virus particles (small arrow) in a rER-derived nearly ribosome free cystern. A Golgi complex is situated immediately next to the rER-cystern.

Replication

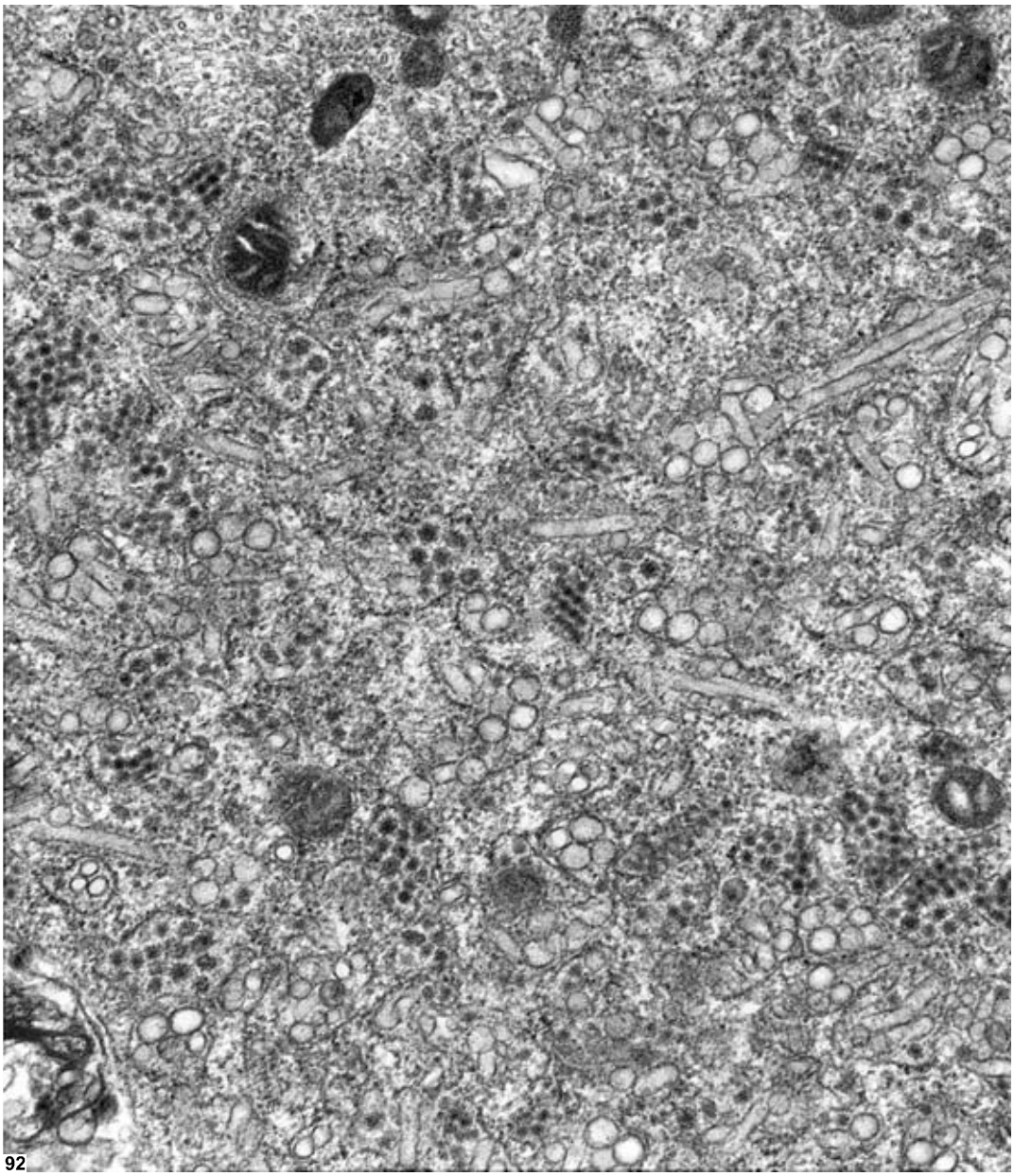


Fig. 92. Dengue-2 virus infected mosquito cell culture. TEM

A dengue-2 positive serum and a dengue-1 convalescent serum were mixed and incubated at 37°C for 30min for virus infection enhancement. The infected cell monolayer was fixed 6 days post-infection and standard processed as described in Fig. 40.

Original magnification: 20,000x. Final magnification: 45,000x.

This late step of cell infection by dengue-2 viruses shows large patches of enlarged cysterns of the rough endoplasmic reticulum inside a syncytium as viral morphogenesis is finishing. Typical and atypical structured virus particles, vesicles and tubules of smooth membrane structures occur together in the same cysterns. Ribosomes are less frequent in these cell regions.

Replication

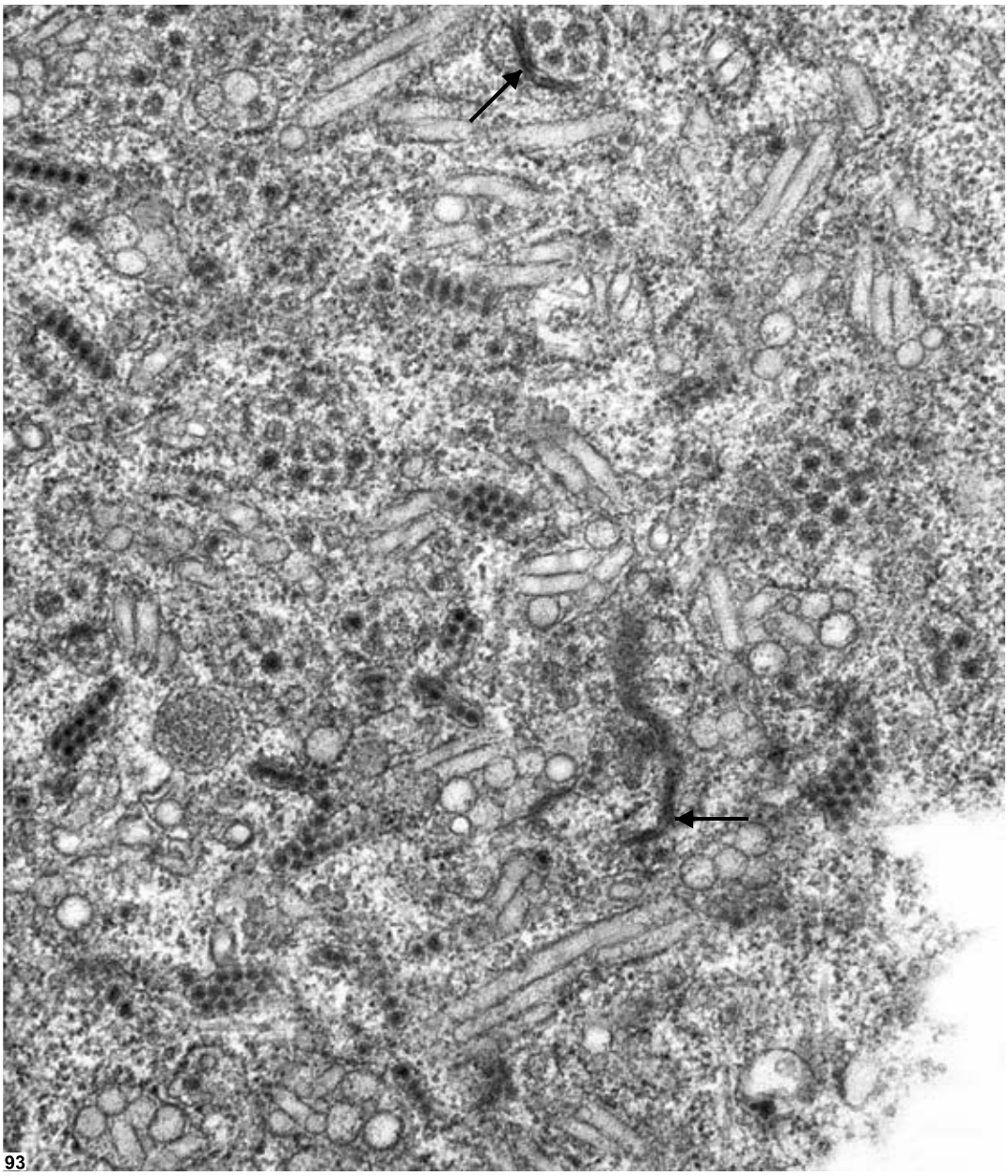


Fig. 93. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture of the 3rd virus passage was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 20,000x. Final magnification: 46,000x.

Typical and atypical structured virus particles are present again as well as smooth membrane structures, inside enlarged cysterns of the rough endoplasmic reticulum. Dense structures (arrows), similar to confronting cysterns (Ghadially, 1982), occur in elder infected cell cultures, when two rER-cysterns are located side by side.

Replication

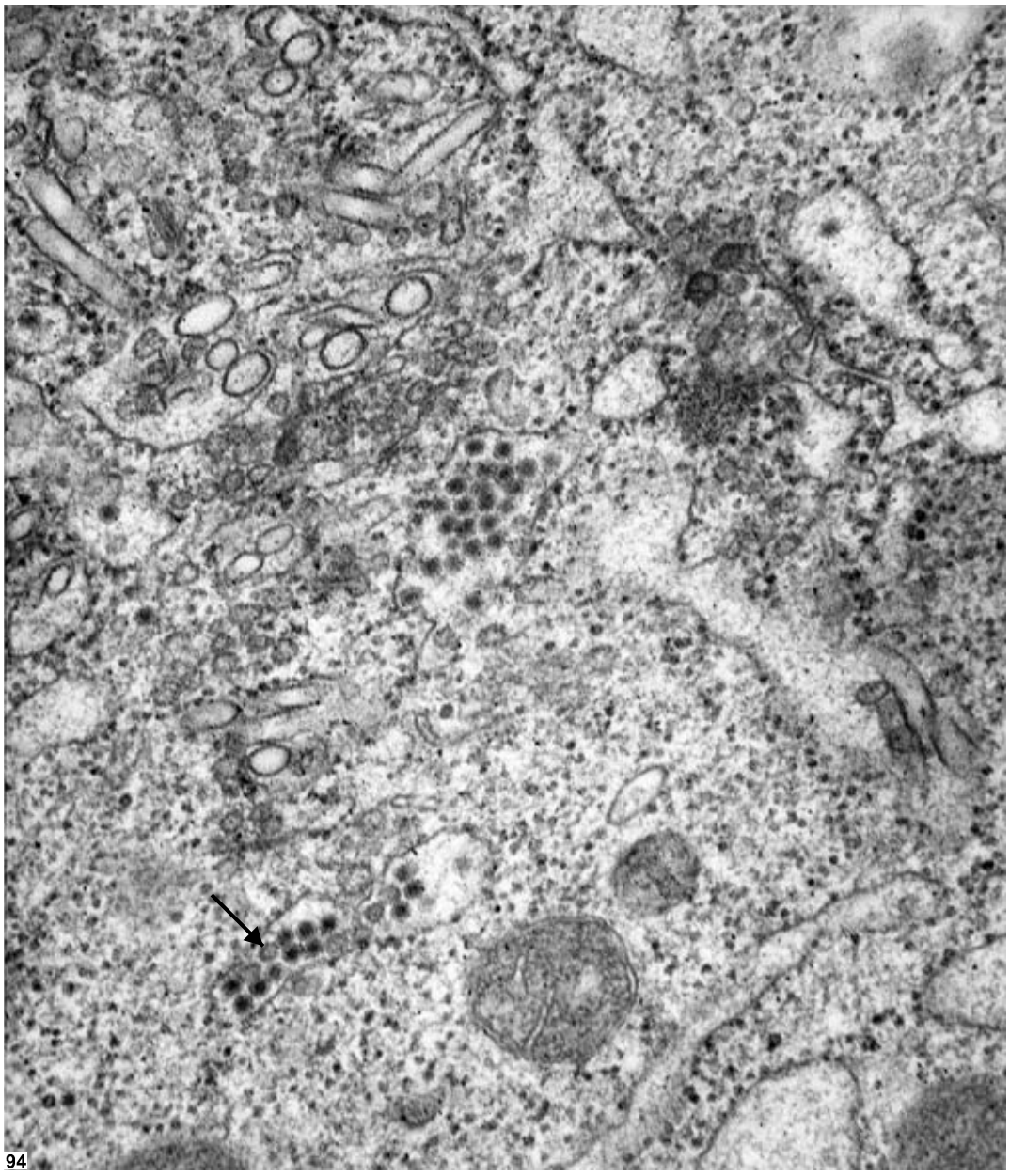


Fig. 94. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 30,000x. Final magnification: 69,000x.

Few RNA-free (arrow) and numerous typical structured virus particles, as well as smooth membrane structures, are detected inside enlarged cysterns of the rough endoplasmic reticulum, which present a light-gray filamentous matrix and few ribosomes.

Replication

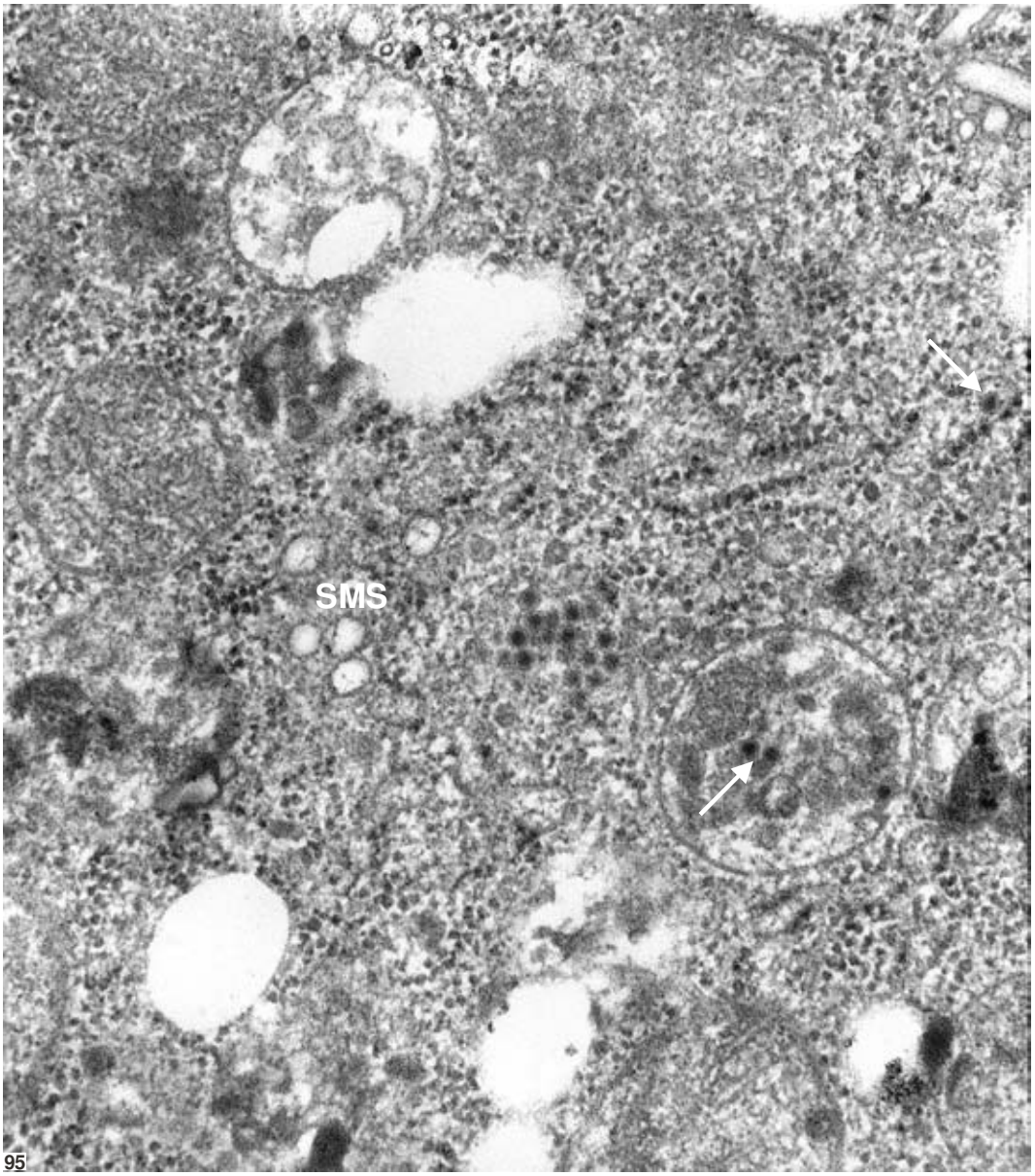


Fig. 95. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 78.

Original magnification: 30,000x. Final magnification: 69,000x.

Typical structured virus particles are assembled inside smooth membrane coated rER-derived ribosome free cysterns. In this figure virus particles (arrows) may be observed inside ribosome coated rER-cysterns, as well as inside a lysosome. Few smooth membrane structures are present.

Maturation and Exocytosis

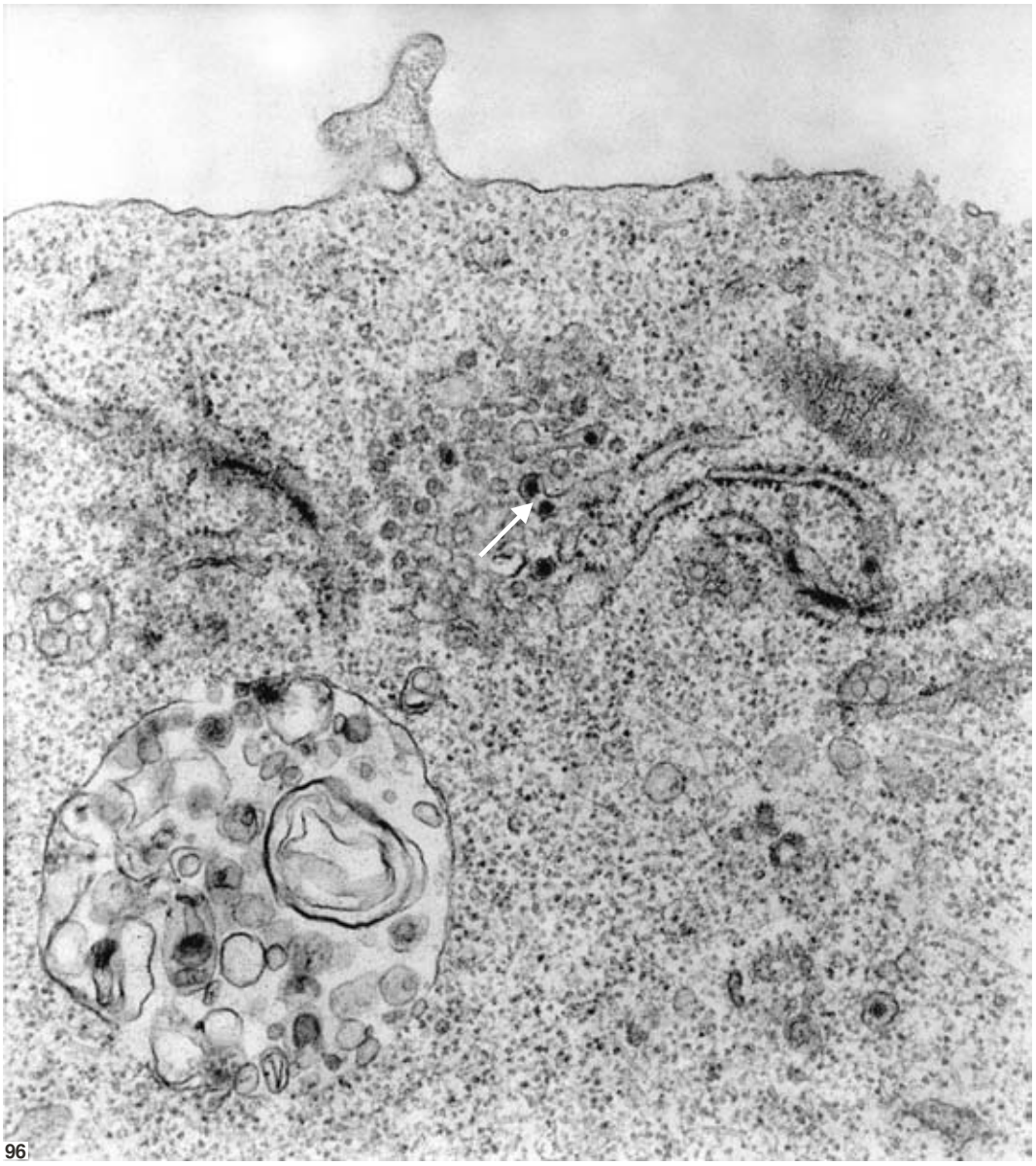


Fig. 96. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 62.

Original magnification: 20,000x. Final magnification: 57,000x.

Virus particles from inside the cysterns of the rough endoplasmic reticulum are being transferred into cysterns of the Golgi complex (arrow).

Maturation and Exocytosis

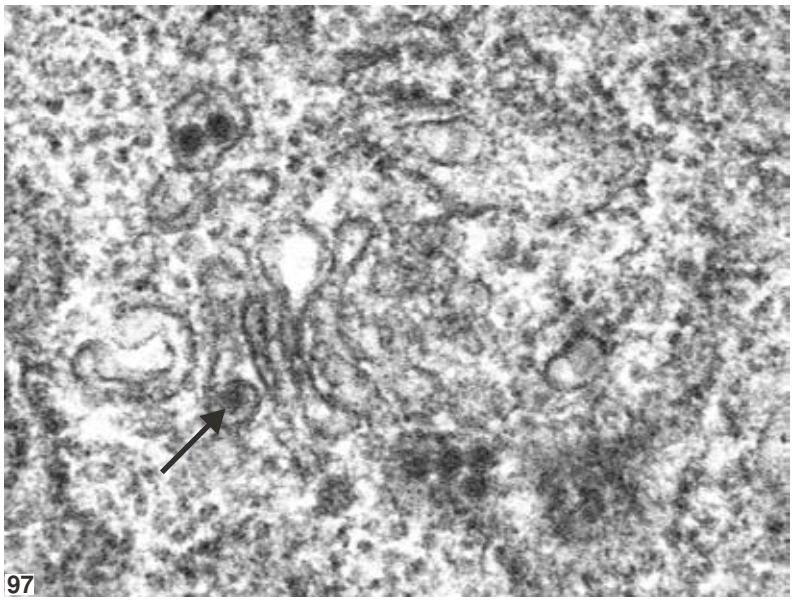


Fig. 97. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 89.

Original magnification: 30,000x. Final magnification: 76,000x.

One (arrow) or more virus particles are found inside vesicles of the Golgi complex. In the lower part of the figure, four virus particles are seen inside a cystern of the rough endoplasmic reticulum.

Maturation and Exocytosis

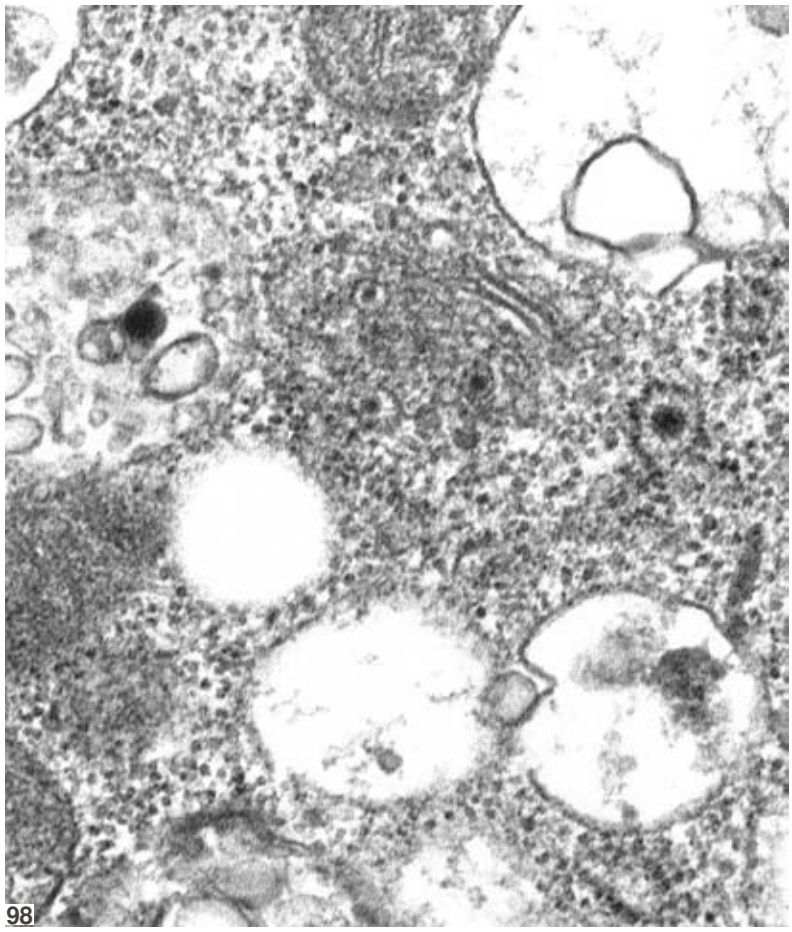


Fig. 98 - Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 78.

Original magnification: 20,000x. Final magnification: 53,000x.

The Golgi complex present typical structured virus particles inside vesicles. A round ribosome coated cystern of the rough endoplasmic reticulum shows one atypical structured virus particle at the middle right of the figure.

Maturation and Exocytosis

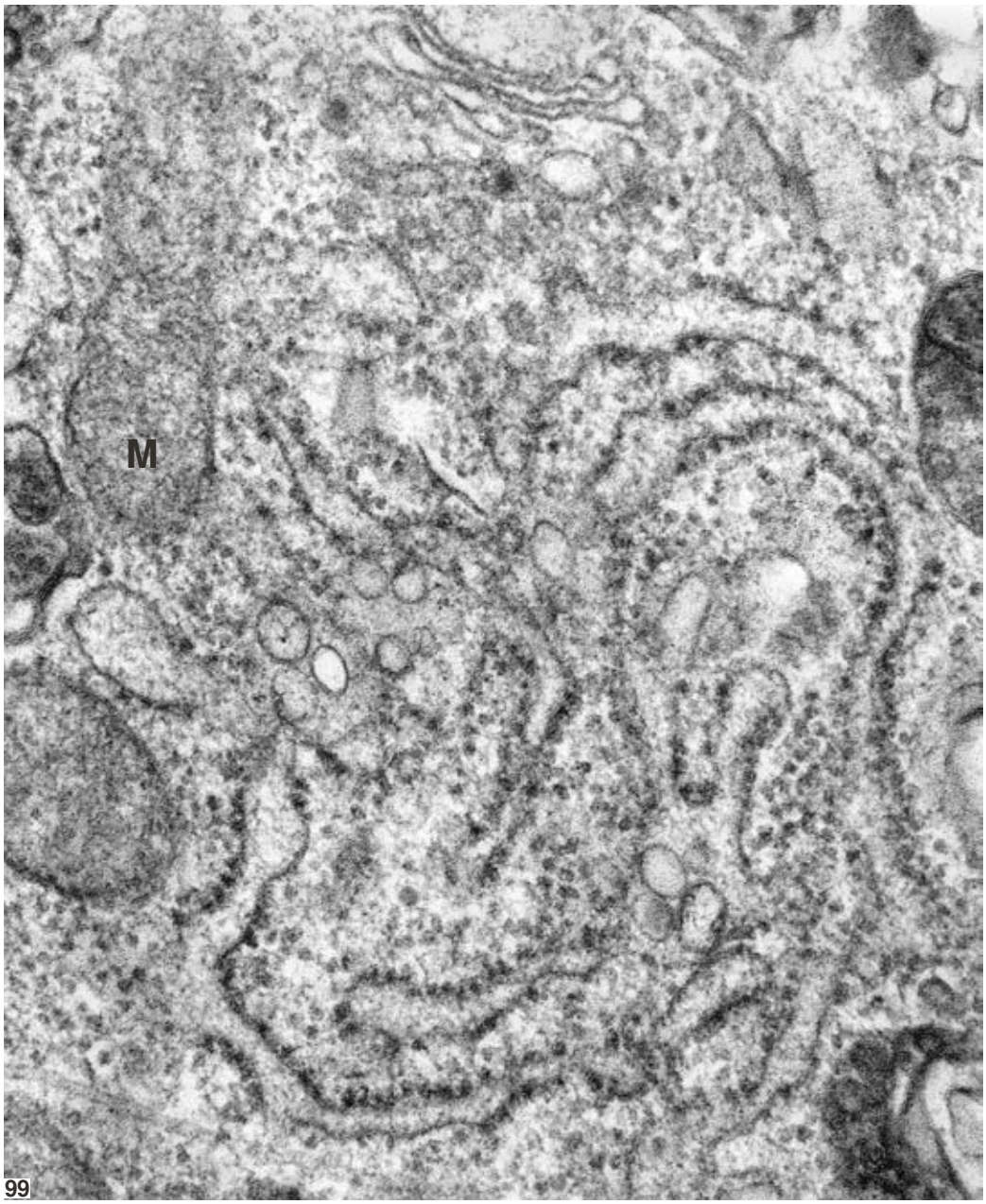


Fig. 99. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 63.

Original magnification: 30,000x. Final magnification: 79,000x.

In the upper side of the figure, typical structured virus particles are enclosed individually in vesicles of the Golgi complex. Below, large cysterns of the rough endoplasmic reticulum present groups of smooth membrane structures inside and enlarged dark stained ribosomes outside.

Maturation and Exocytosis

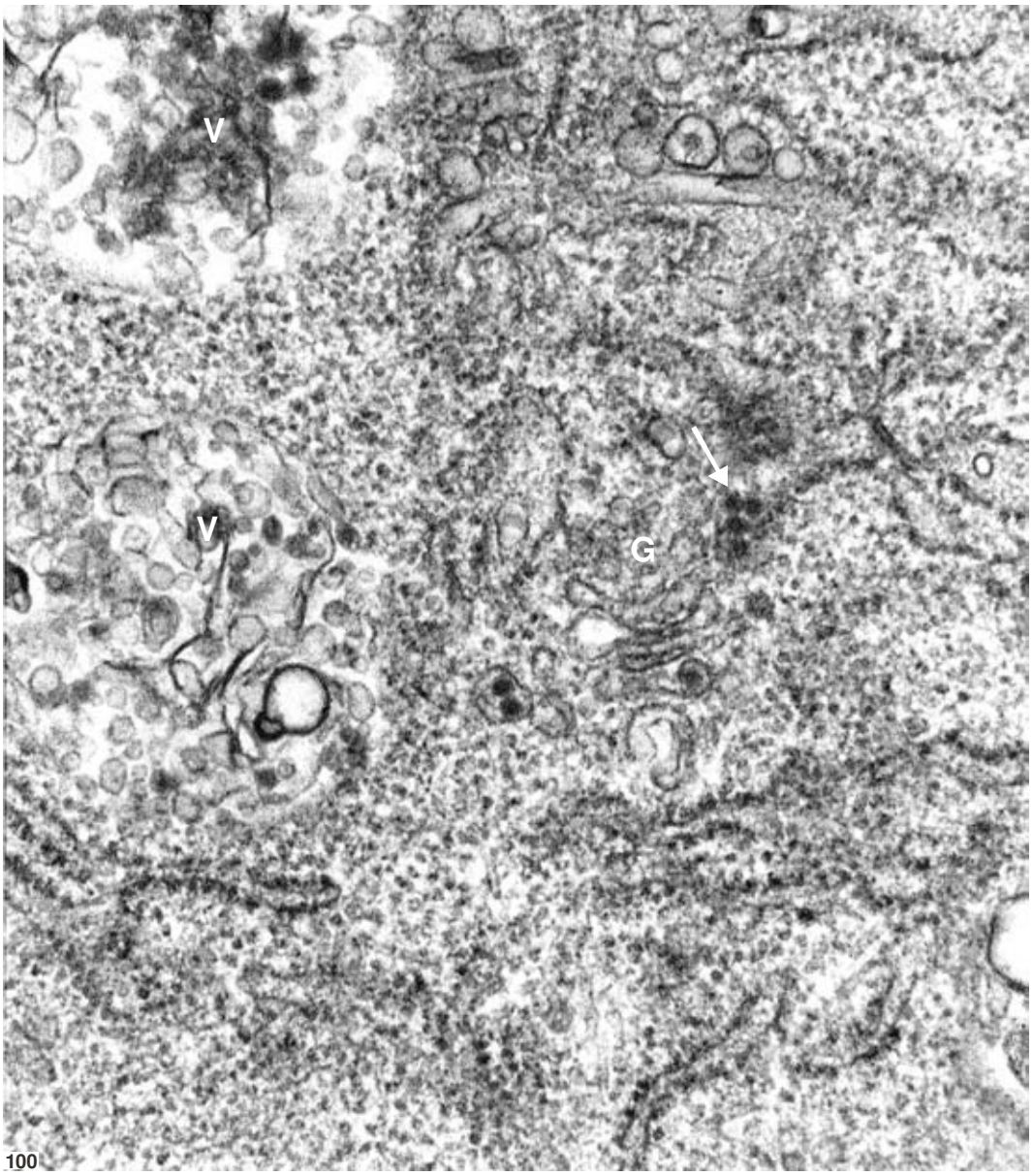


Fig. 100. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 89.

Original magnification: 30,000x. Final magnification: 70,000x.

Lower magnification of Fig. 97, comprising a larger area of the virus infected cell. Enlarged cysterns of the rough endoplasmic reticulum (arrow) show virus particles directed toward the Golgi complex. Above, another cystern of the rough endoplasmic reticulum presents tubules and vesicles of smooth membrane structures inside. Vacuoles containing numerous different sized vesicles, as well as virus particles (the upper vacuole) are frequently found inside the infected cells and syncytia, during this stage of virus morphogenesis.

Maturation and Exocytosis

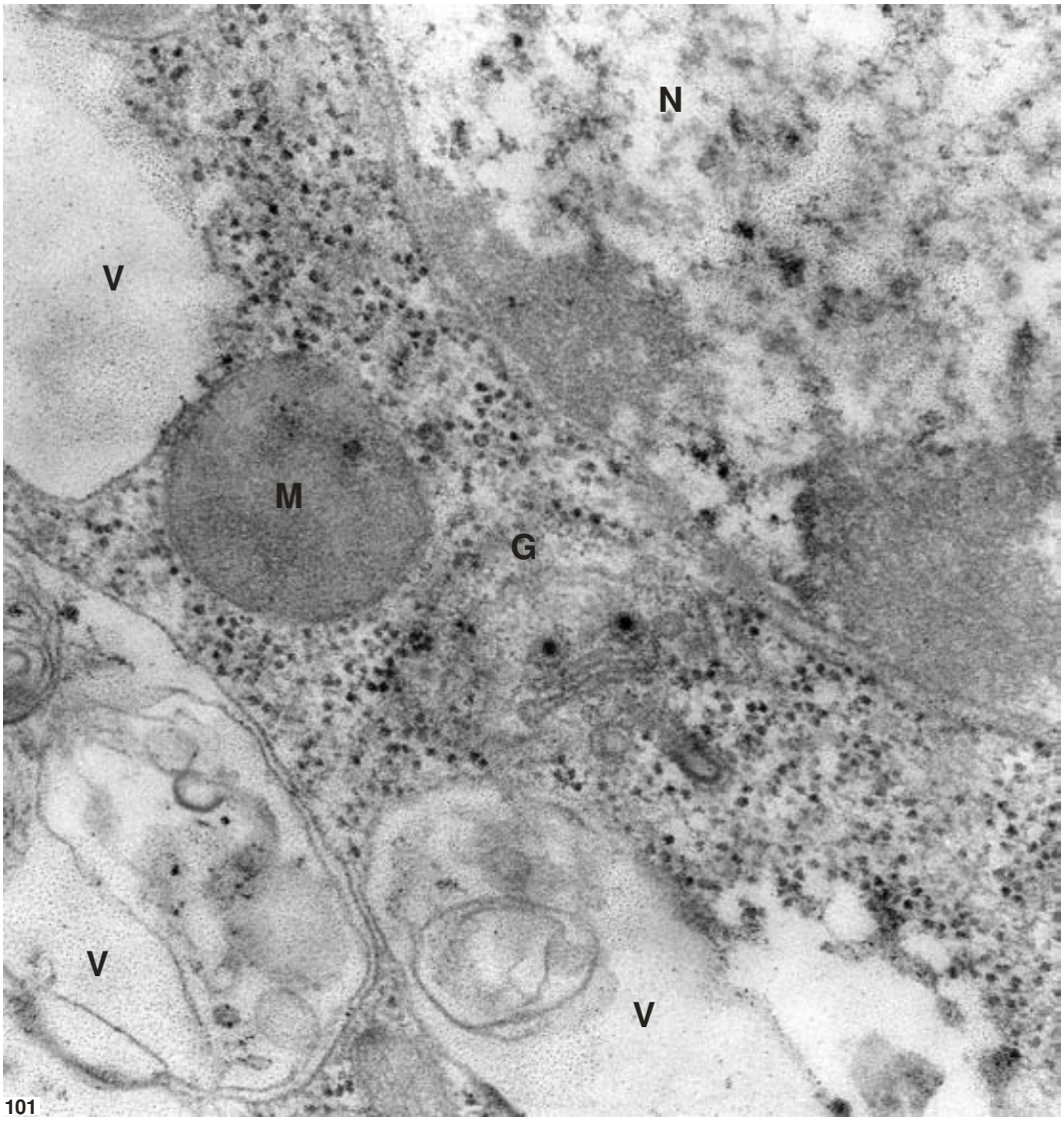


Fig. 101. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.

Original magnification: 32,900x. Final magnification: 80,000x.

This ultrathin section, harvested on a collodion coated grid, was processed for EDTA-treatment as described in Fig. 67.

The Golgi complex in the center of the figure (like a duck's face) presents two virus particles, each inside a vesicle. The nuclear DNA was unstained, as the uranyl stain was extracted by the EDTA-treatment. Nevertheless, the two virus particles are not affected by this treatment and remain dark stained, confirming the RNA-nature of the nucleocapsids.

Maturation and Exocytosis

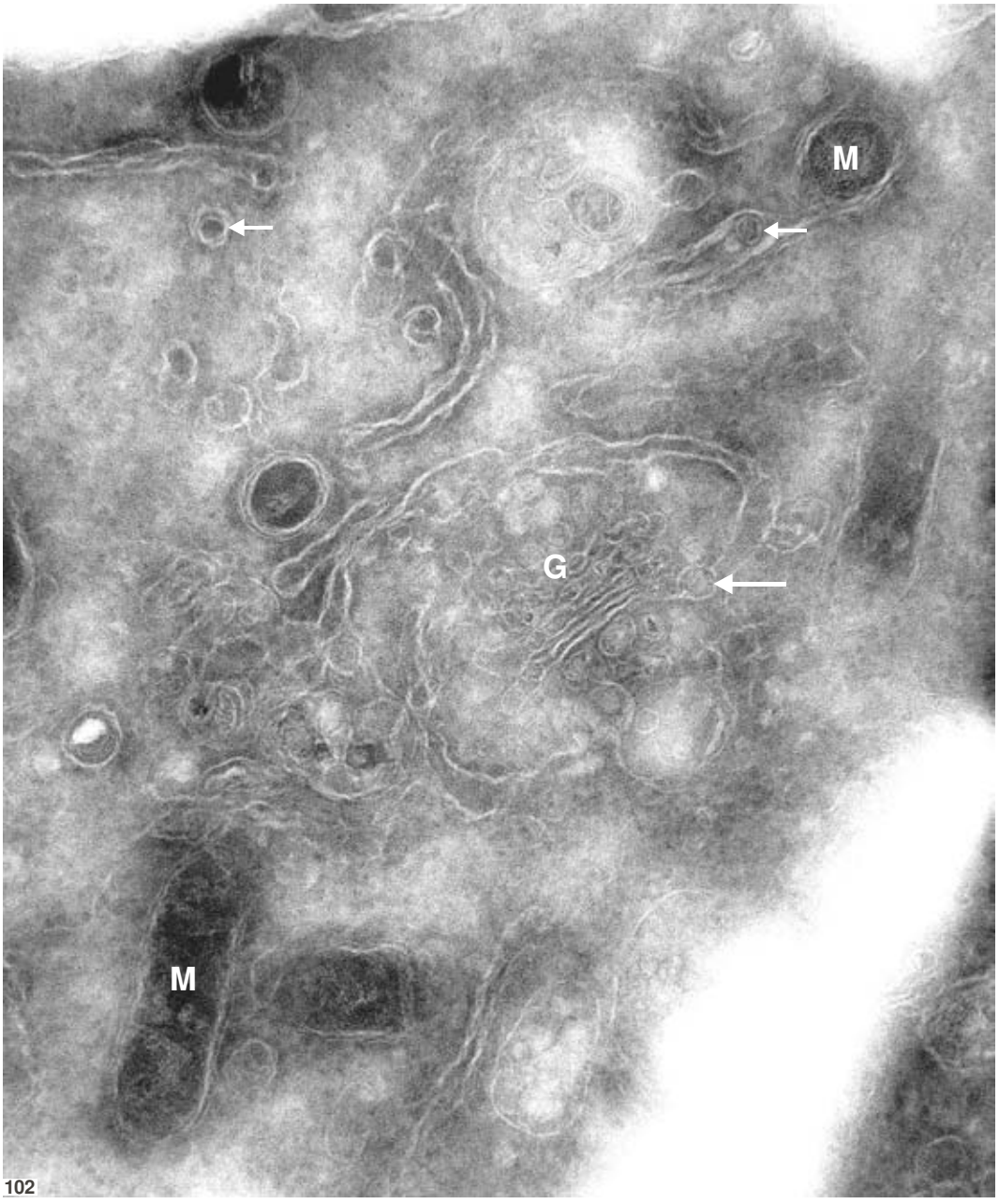


Fig. 102. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 80.

Original magnification: 30,000x. Final magnification: 85,000x.

Cryosection. Mitochondria are dark stained, membranes are unstained. A Golgi complex shows cysterns and vesicles containing virus particles in the center of the figure. Ribosomes are not detectable by this technique. Vesicles of the smooth membrane structures (short arrows) are dark stained, virus particles are gray (long arrow).

Maturation and Exocytosis

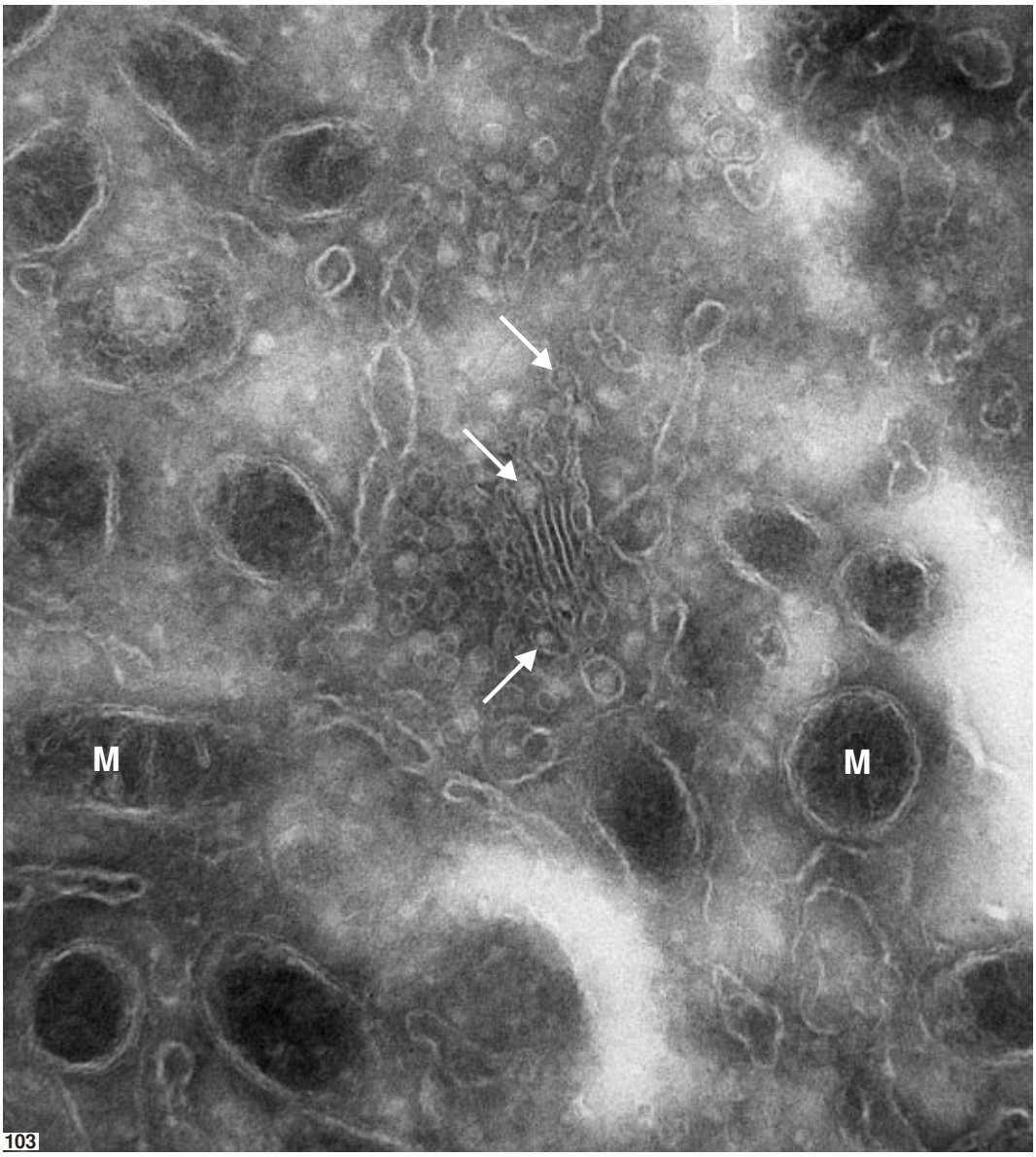


Fig. 103. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 80.

Original magnification: 30,000x. Final magnification: 73,000x.

Similar area as in Fig. 102 at higher magnification. Mitochondria are dark stained. Membranes of the rough endoplasmic reticulum and of the mitochondria are light. The ribosomes of the rough endoplasmic reticulum are not visible. A Golgi complex in the center of the figure shows cysterns and vesicles containing virus particles (arrows).

Inactivation: cell lysis

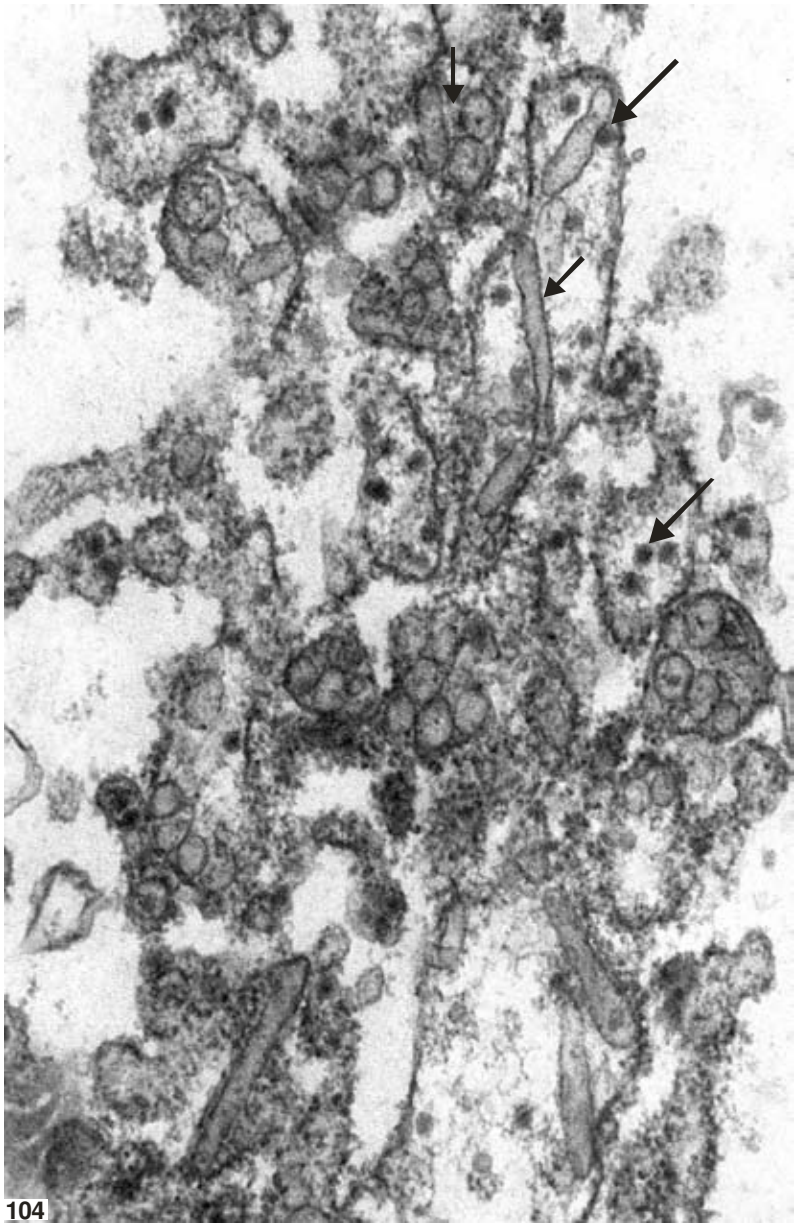


Fig. 104. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 92.

Original magnification: 20,000x. Final magnification: 55,000x.

This figure shows organells of a disrupted syncytium. After a strong vacuolization due to the virus infection, the cell membrane of a syncytium open up and the virus particles (long arrows) and smooth membrane structures (small arrows) containing cysterns of the rough endoplasmic reticulum remain free. Virus particles remain enclosed inside vesicles.

Inactivation: cell lysis

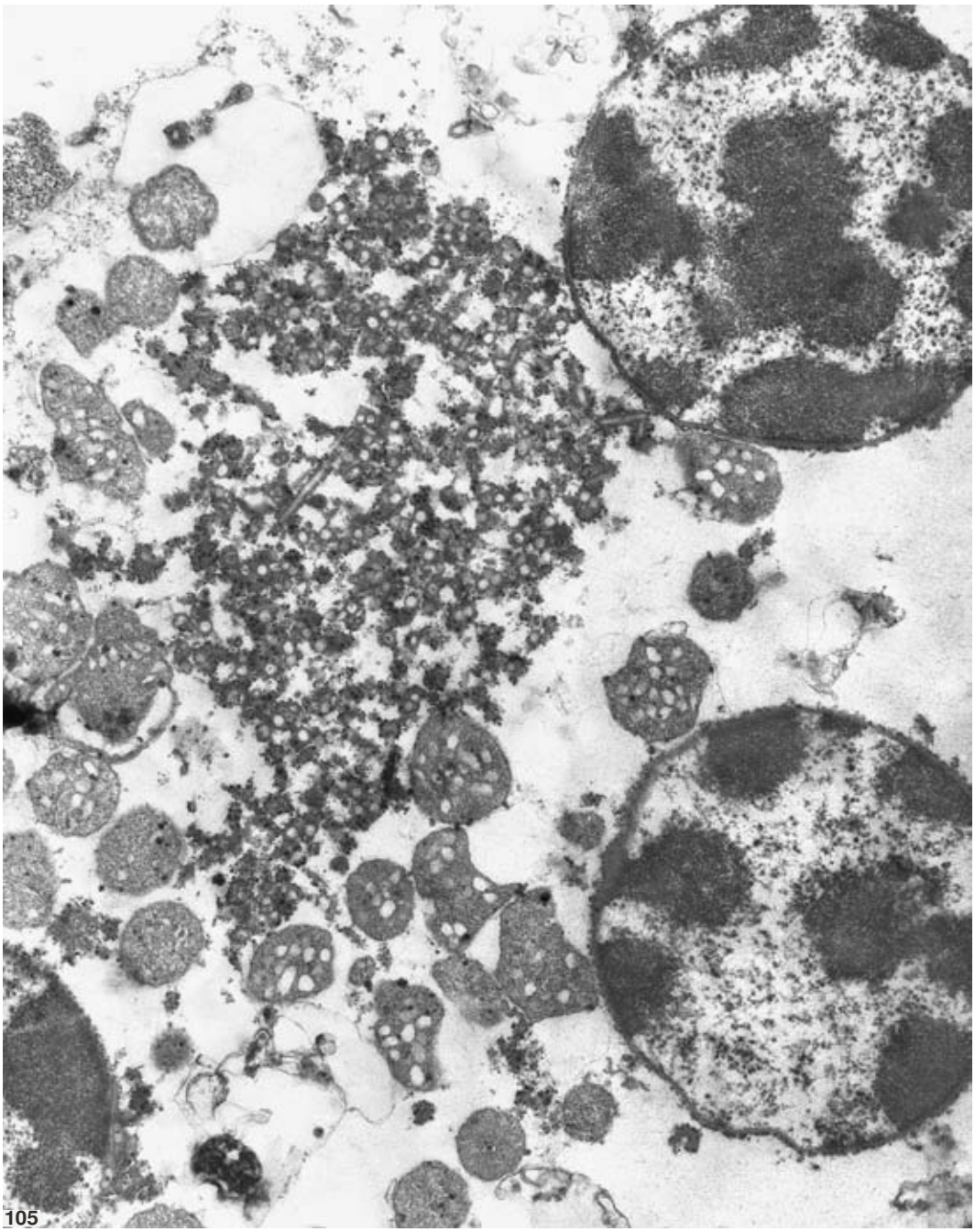


Fig. 105. Dengue-1 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 44.
Original magnification: 8,000x. Final magnification: 21,000x.
Three nuclei of a totally damaged syncytium, modified mitochondria and a patch in the center of the figure, where virus morphogenesis had been occurred, are still recognized.

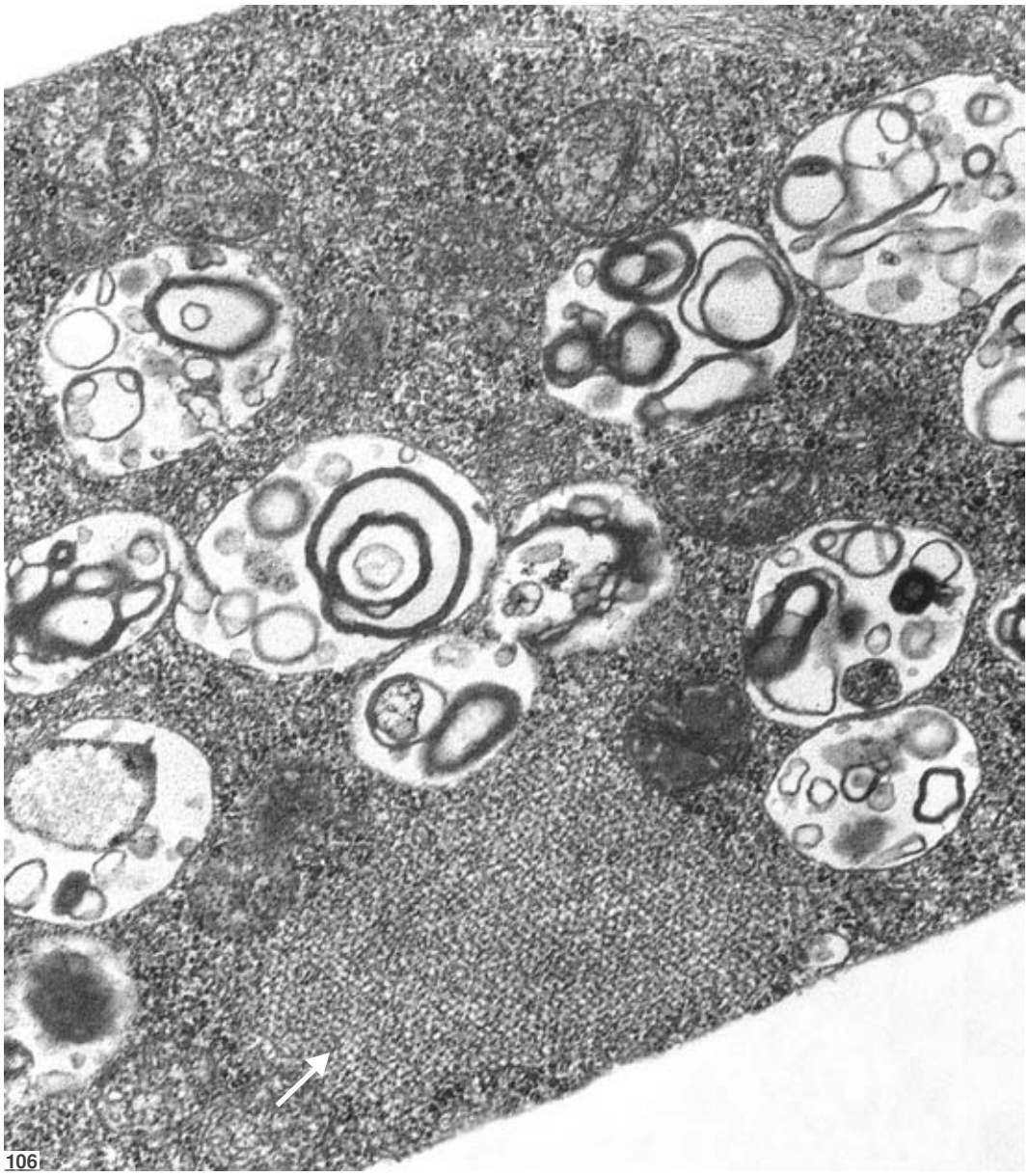


Fig. 106. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 92.

Original magnification: 20,000x. Final magnification: 46,000x.

This infected syncytium shows numerous lysosomes, modified mitochondria and a large proteinous crystal (arrow), presenting subunits disposed in paracrystalline arrays.

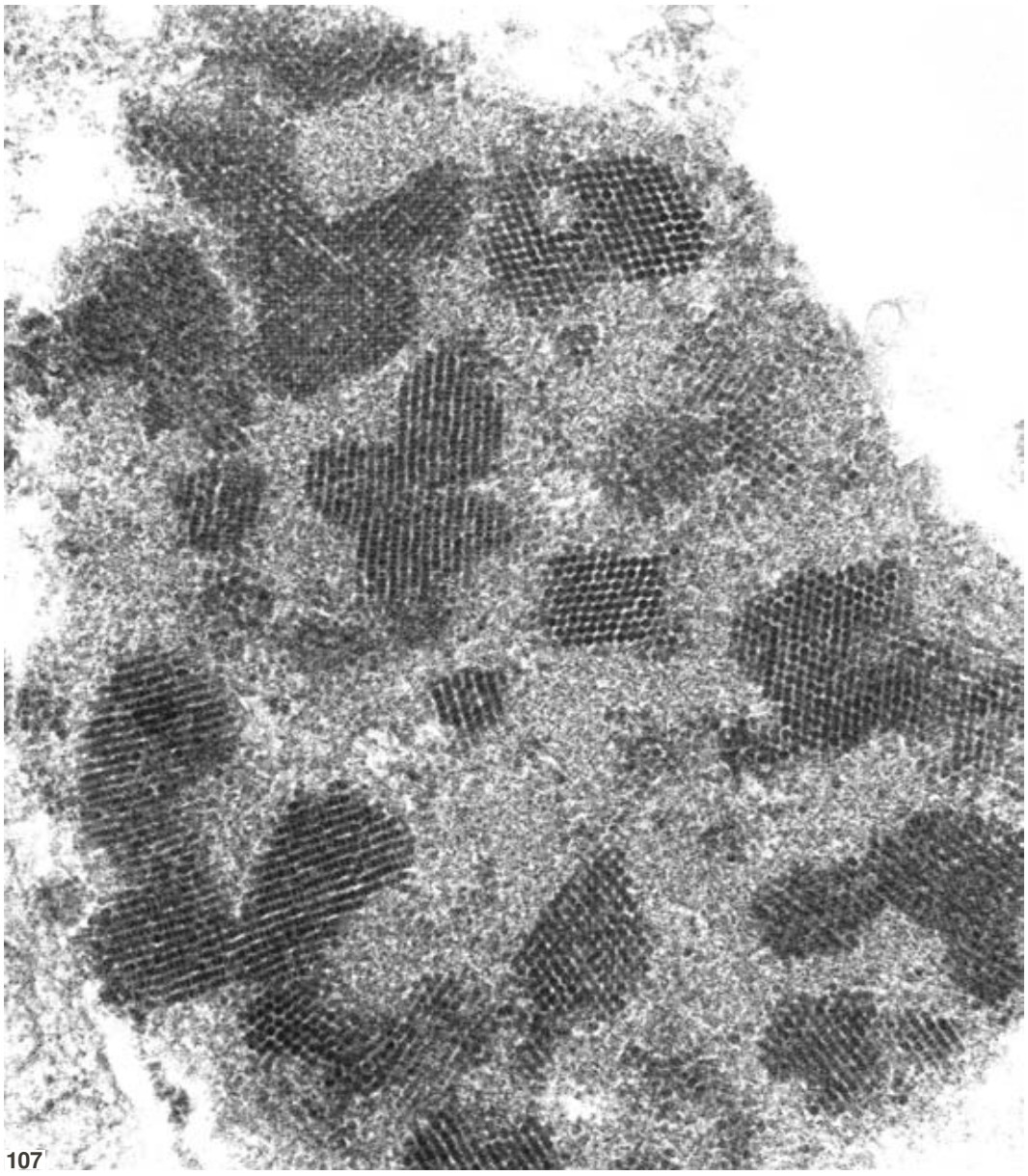


Fig. 107. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 58.

Original magnification: 30,000x. Final magnification: 67,000x.

Paracrystalline arranged proteinous subunits are composed of numerous protein crystals, inside the cytoplasm of an infected mosquito cell. These crystals are supposed to be a result of the metabolic activity of the virus infected cell (Ko *et al*., 1979).

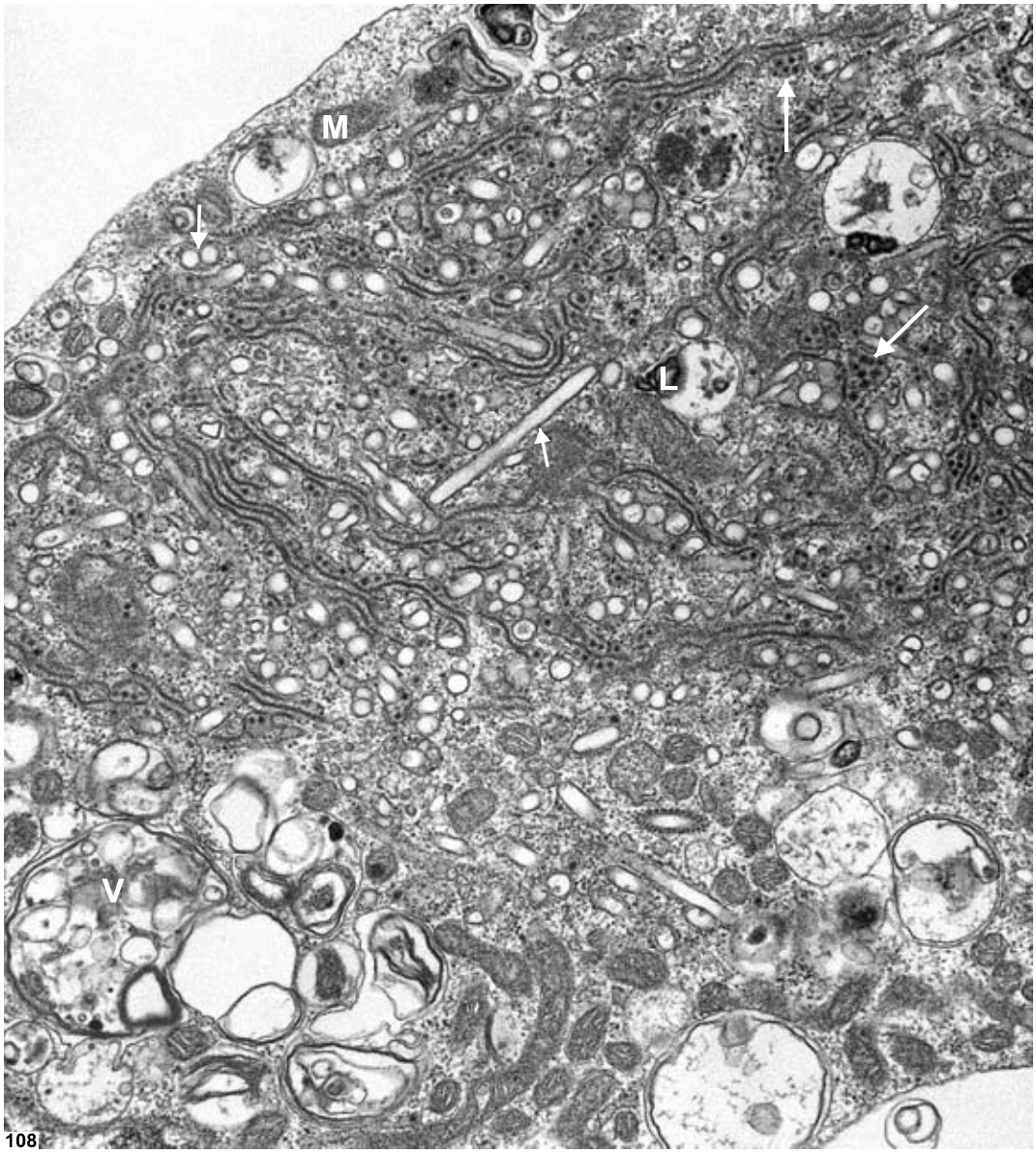


Fig. 108. Dengue-1 virus infected mosquito cell culture. TEM

The cell culture was fixed 6 days post-infection and standard processed as described in Fig. 40.

Original magnification: 12,000x. Final magnification: 29,000x.

During virus morphogenesis, the rough endoplasmic reticulum developed intensely inside a syncytium. Like in previous stages of the cell infection (Fig. 67), virus particles (long arrows) and smooth membrane structures (small arrows) containing cysterns are located in regions of the cell cytoplasm where mitochondria, nuclei, lysosomes and vacuoles are almost absent. The numerous cysterns of the rough endoplasmic reticulum are frequently disposed in parallel (confronting cysterns). The contact faces of adjacent cysterns are strongly contrasted, as well as the viral nucleocapsids. Lysosomes are containing also viral particles and vacuoles are full of cell dejects. (compare with Figs. 79, 93, 110-112)

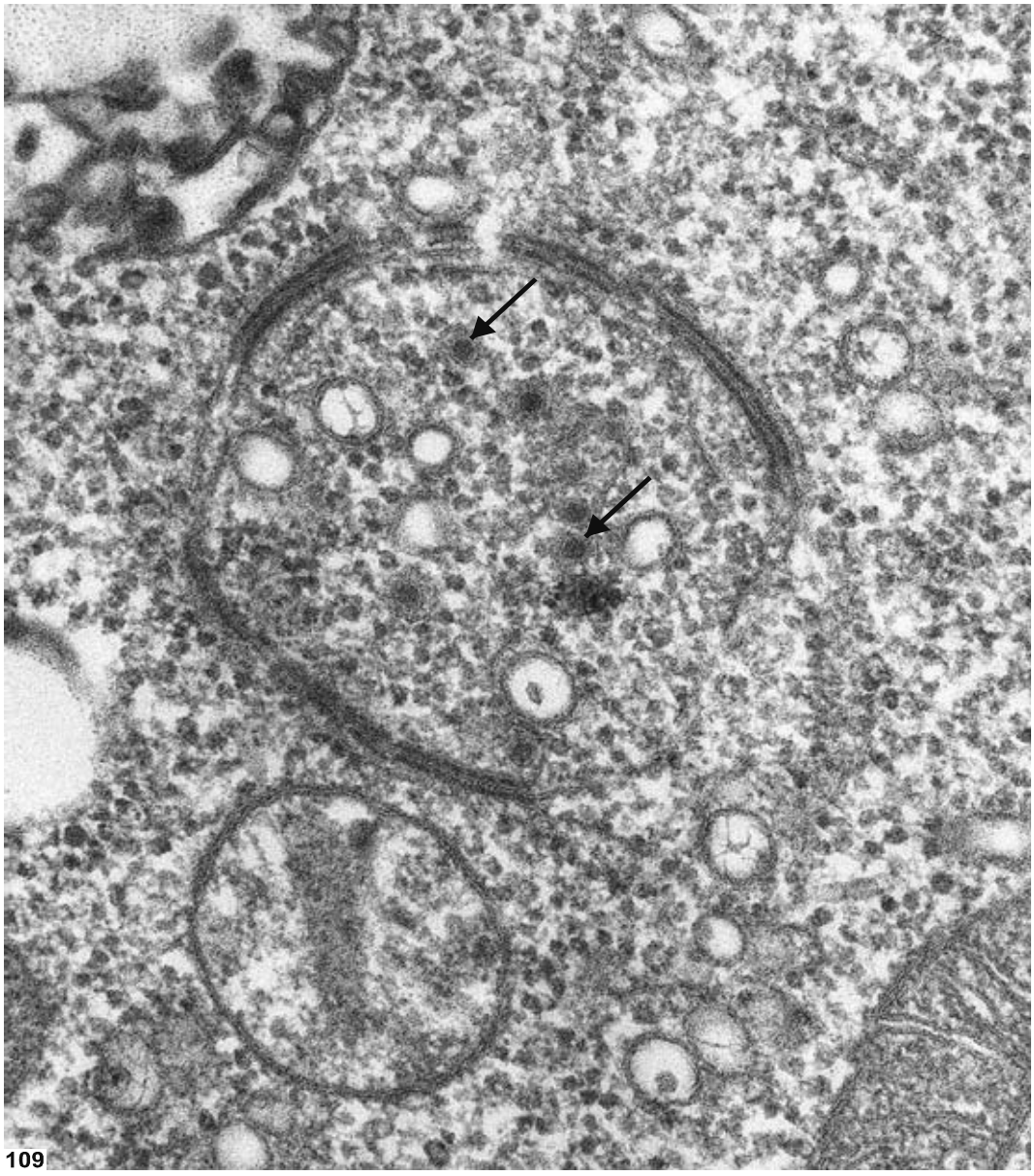


Fig. 109. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 78.

Original magnification: 30,000x. Final magnification: 77,000x.

The fourth passage of the virus in mosquito cell culture shows the formation of “double-track-structures” (Ko *et al.*, 1979) inside an “old” syncytium, of similar appearance as the “confronting cysterns” (Ghadially, 1982).

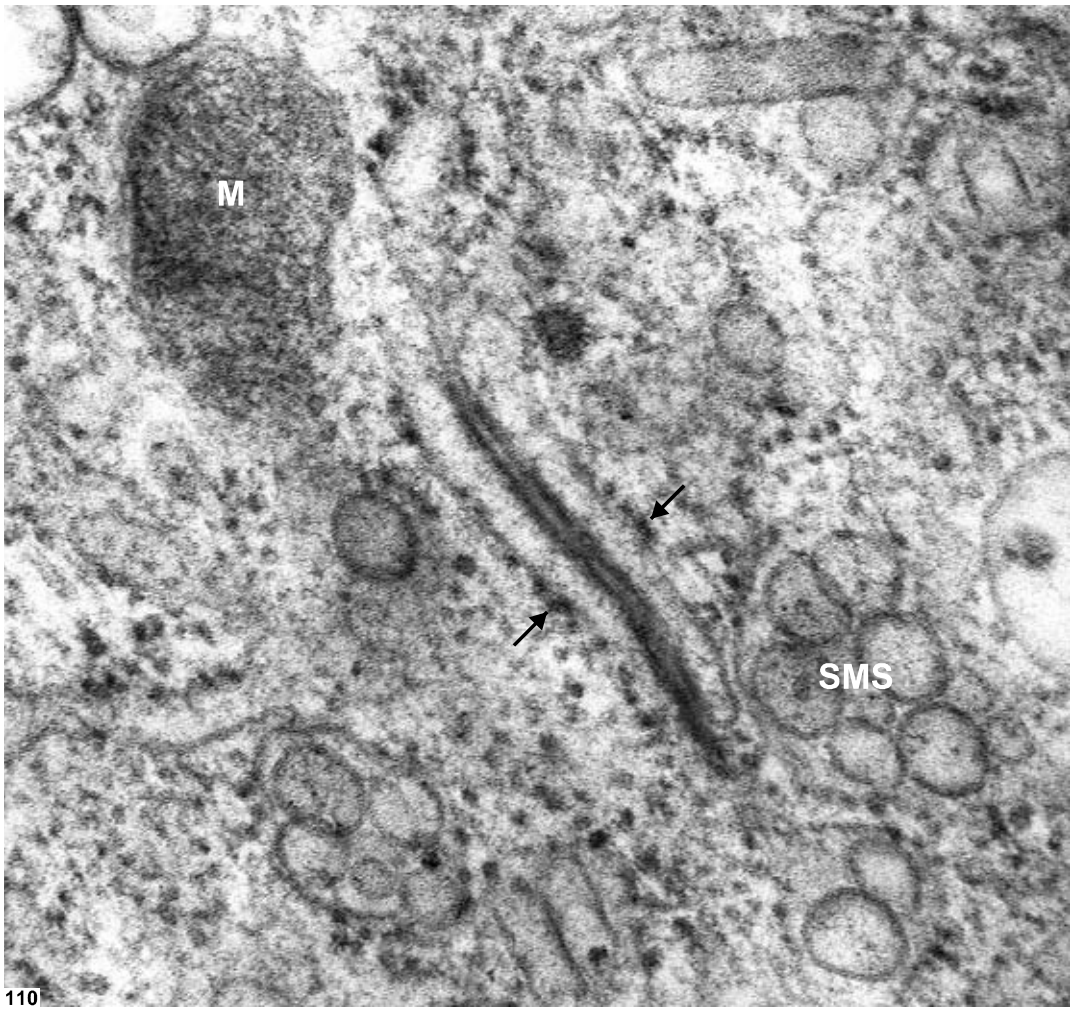


Fig. 110. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 50,000x. Final magnification: 125,000x.

High magnification of a “double-track-structure”. Two cysterns of the rough endoplasmic reticulum are disposed in parallel, ribosomes (arrows) are seen outside the rER-membranes. A dark stained material between the two cysterns is periodically interrupted by lighter regions. A very thin dark filament occurs in the middle part of this structure, as may be observed also in Fig. 109. The presence of these structures is associated to the virus infection of the cell. Smooth membrane structures are also frequent in this part of an infected cell.

Special Features

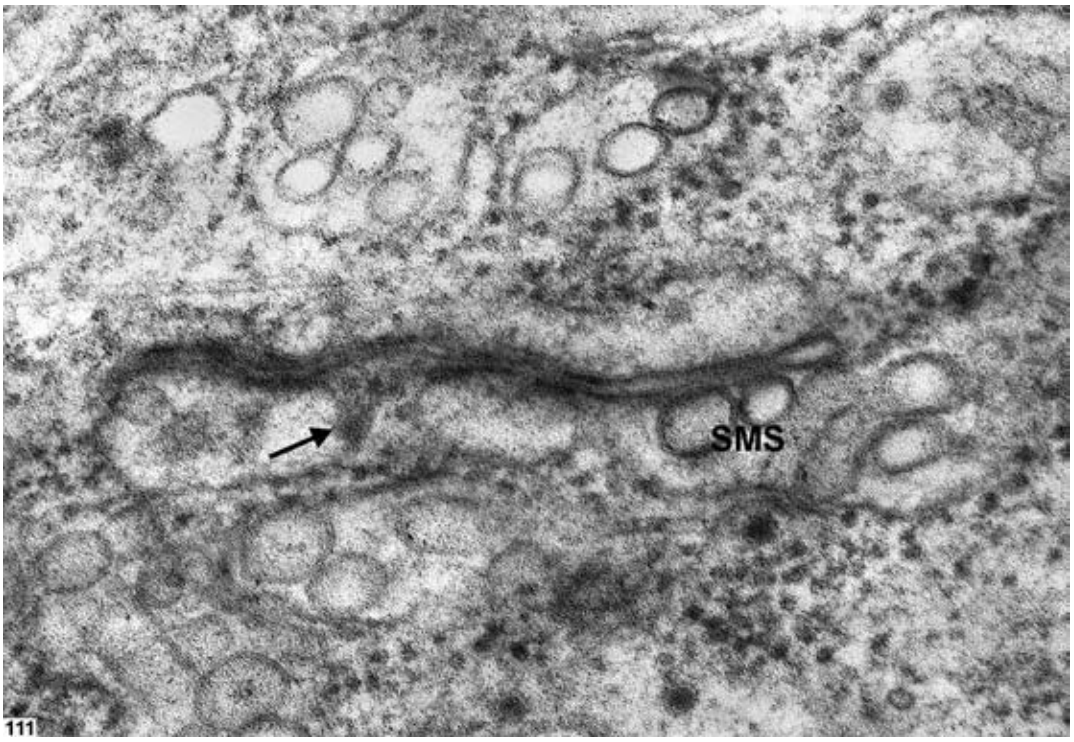


Fig. 111. Dengue-2 virus infected mosquito cell culture. TEM

The same preparation as in Fig. 110.

Original magnification: 50,000x. Final magnification: 125,000x.

Two confronting cysterns of the rough endoplasmic reticulum form a “double-track-structure” inside a syncytium. The upper one shows no special structures. The lower one shows one virus particle (arrow) and several smooth membrane structures. Another cystern below shows also one typical structured virus particle and smooth membrane structures. Several cysterns of the rough endoplasmic reticulum and a microtubule are represented in the upper part of the figure.

Special Features

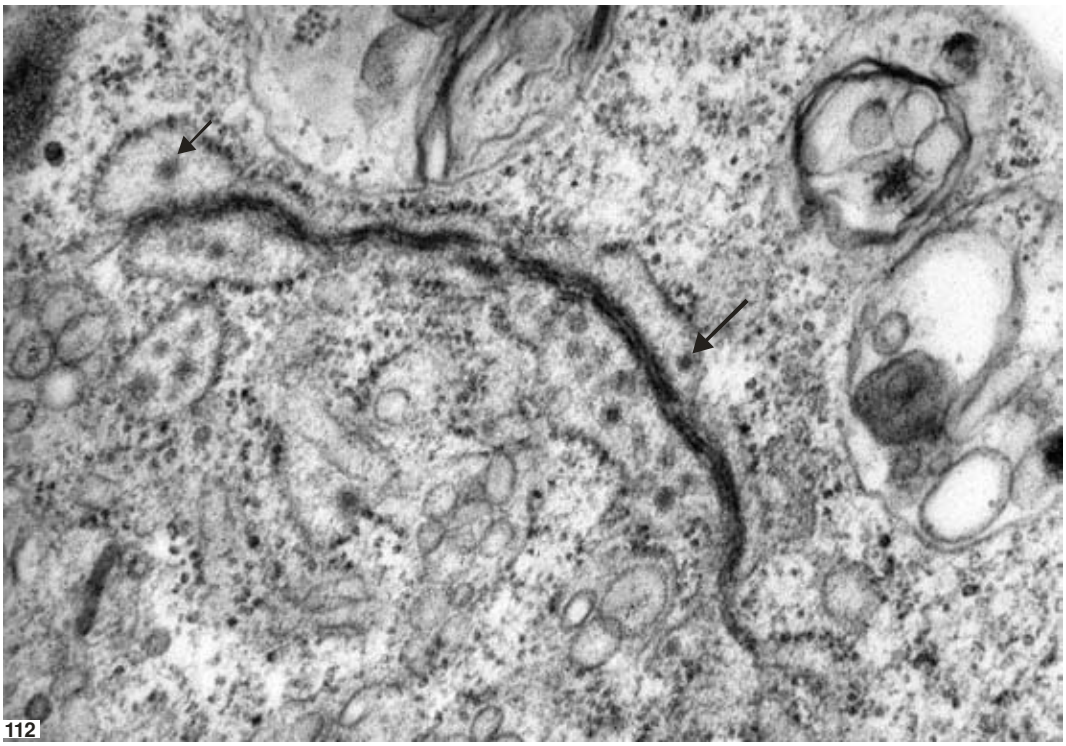


Fig. 112. Dengue-2 virus infected mosquito cell culture. TEM

The similar preparation as in Fig. 110 using another patient serum as inoculum.

Original magnification: 30,000x. Final magnification: 87,000x.

A very long, periodically interrupted “double-track-structure” shows two confronting cysterns of the rough endoplasmic reticulum, which contain some typical (long arrow) and numerous atypical (small arrow) structured virus particles.

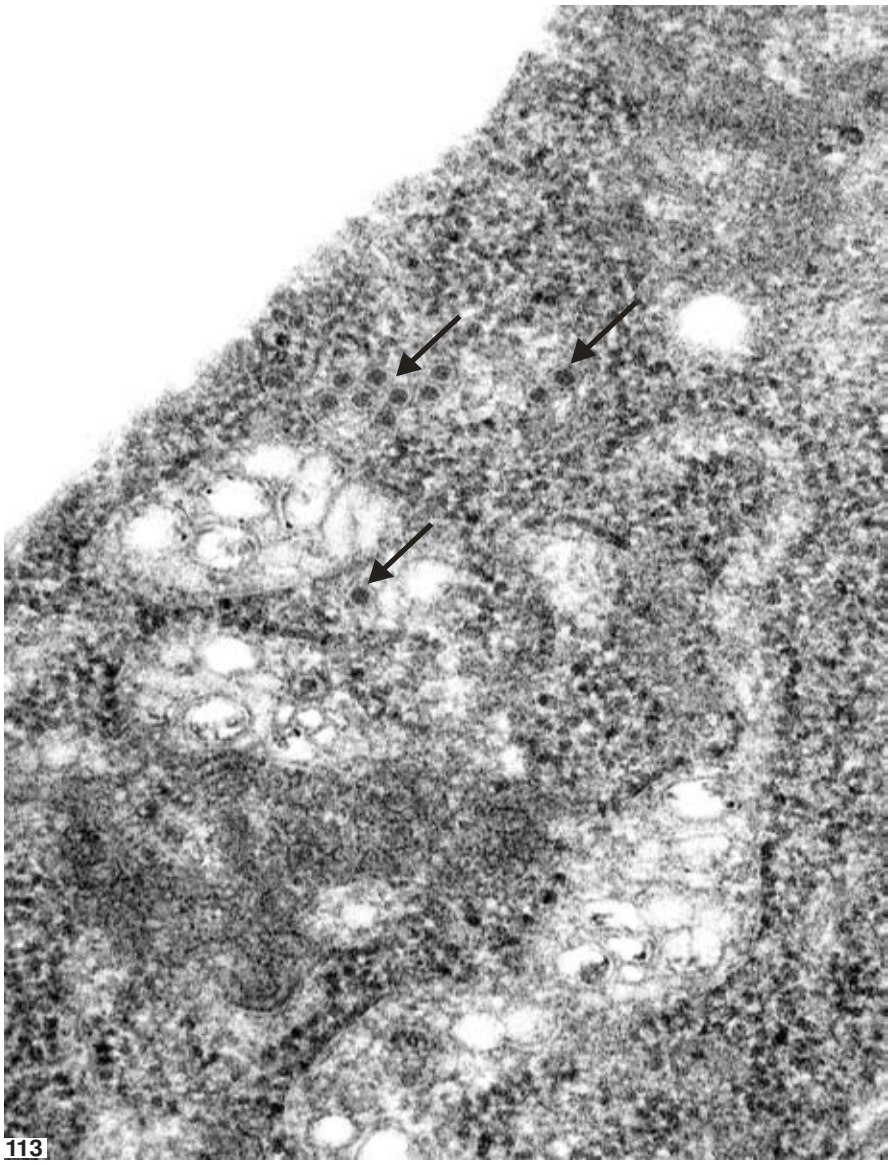


Fig. 113. Dengue-2 virus infected mosquito cell culture. TEM

The cell culture was fixed 3 days post-infection with glutaraldehyde only and low temperature embedded in LR-Gold resine (Grief *et al*., 1997). Ultrathin sections were double stained with uranyl acetate and lead citrate.

Original magnification: 30,000x. Final magnification: 79,000x.

LR-Gold resin is responsible for this different aspect of a dengue virus infected mosquito cell. Cell membranes are indistinct. Smooth membrane structures are nearly “empty”. Virus particles show a very dark core and a lighter envelope (long arrows). Free ribosomes and rER-bound ones are strongly stained.

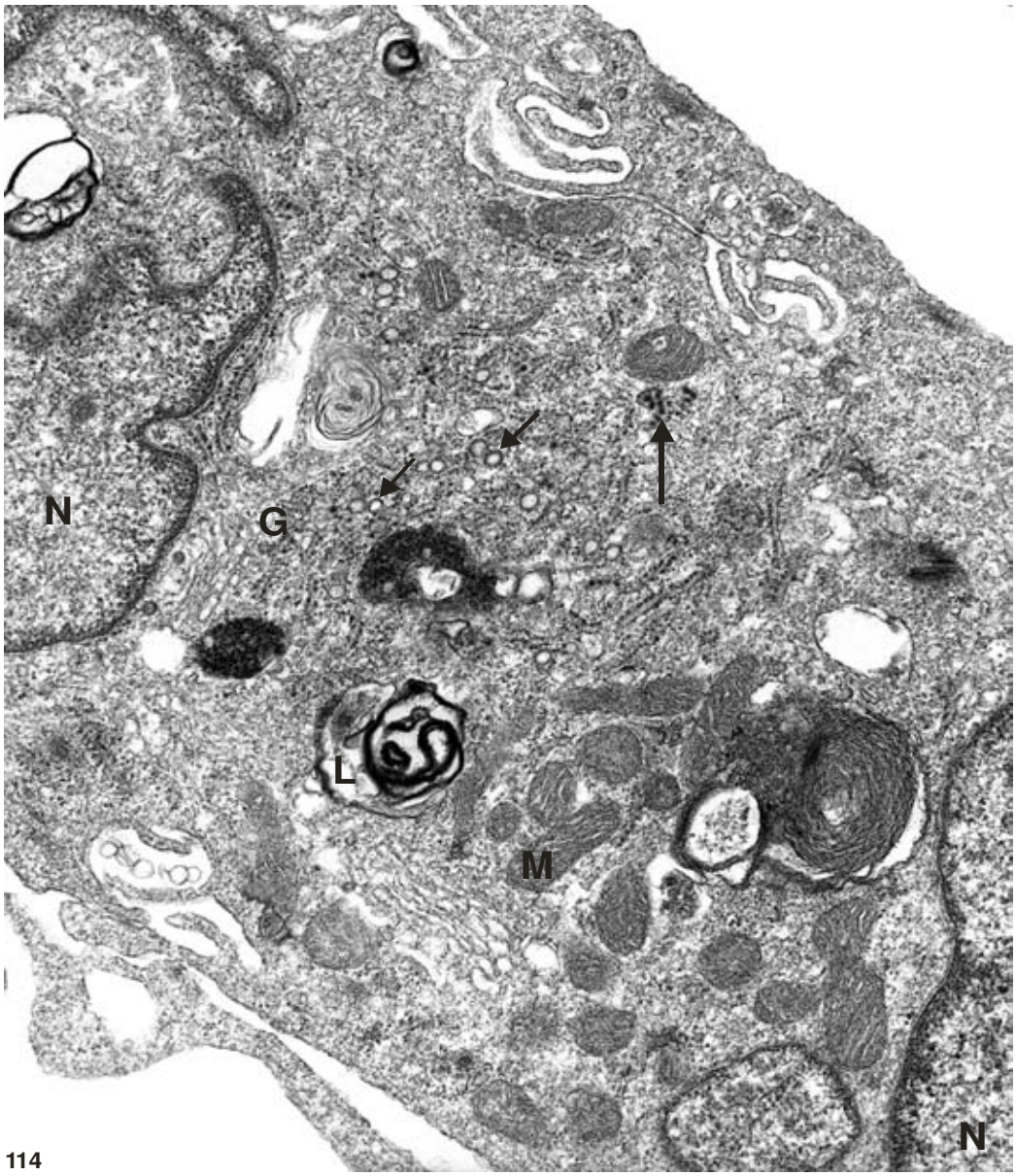


Fig. 114. Dengue-2 virus infected Vero cell culture. TEM

Vero cell culture was fixed 3 days post-infection and standard processed as described in Fig. 40.

Original magnification: 12,000x. Final magnification: 26,000x.

Dengue viruses replicate also in vertebrate cell lines. Nevertheless the quantity of virus particles and smooth membrane structures is lower than in mosquito cell cultures. A region in the middle of this figure of an infected Vero cell, where virus (long arrow) morphogenesis is in progress, can be detected. Although two nuclei are present, syncytia formation is uncommon. Smooth membrane structures (small arrows) are also present inside cysterns of the rough endoplasmic reticulum.

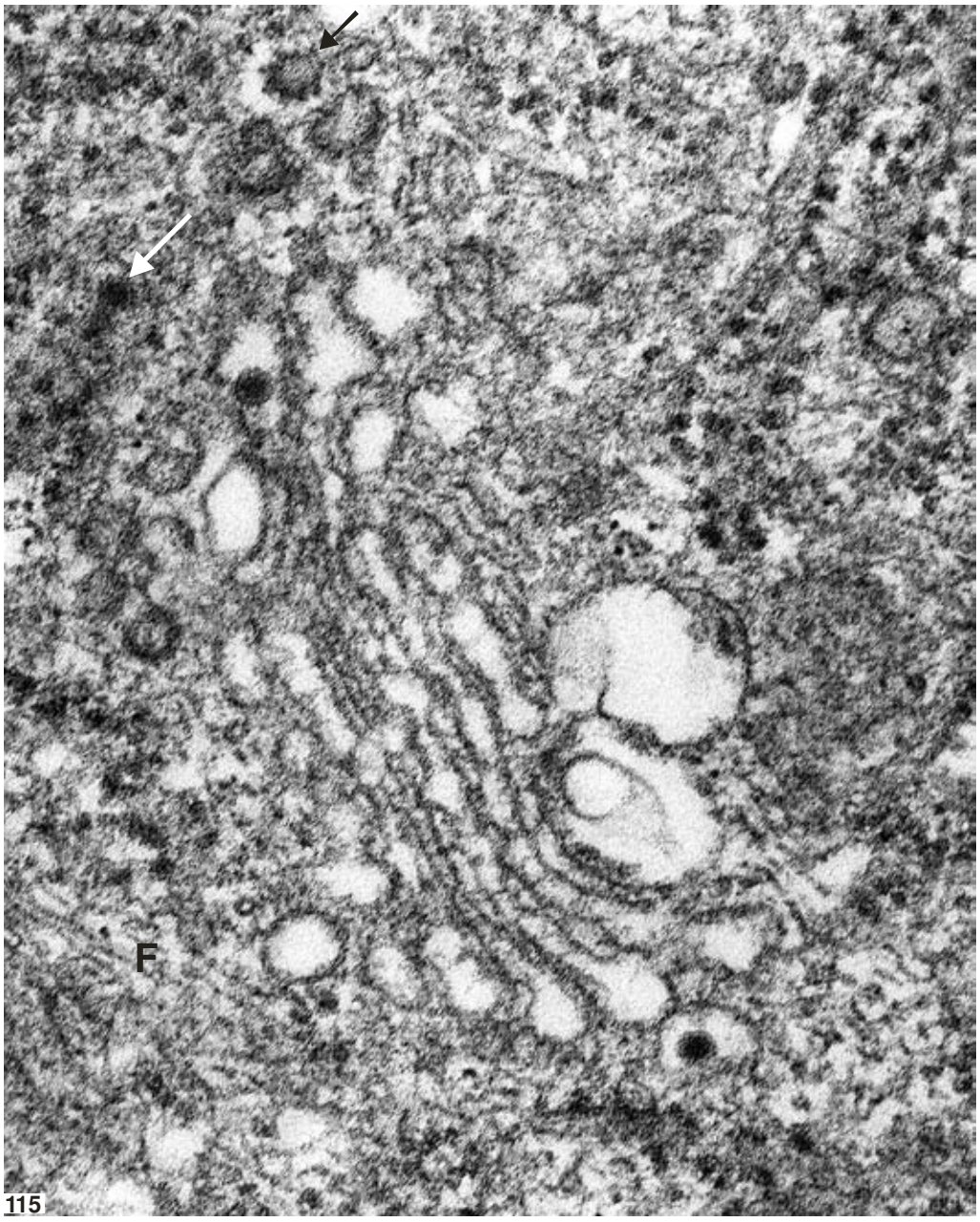


Fig. 115. Dengue-2 virus infected Vero cell culture. TEM

A similar preparation as in Fig. 114.
Original magnification: 50,000x. Final magnification: 118,000x.
The infected Vero cell shows a Golgi complex with cysterns and vesicles. Two darkly stained virus particles are being transported, each one inside a vesicle, toward the cell membrane for exocytosis. Another virus particle inside a cystern of the rough endoplasmic reticulum is ready to be transferred into the Golgy complex (long arrow). This is the active way of dengue virus processing. Clathrin-coated vesicles (small arrow) and filaments of the cytoskeleton may be easily distinguished in Vero cells.

THE END