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REPLICATION OF DENGUE VIRUSES IN MOSQUITO CELL CULTURES – A MODEL FROM ULTRASTRUCTURAL OBSERVATIONS

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Mosquito cell cultures infected with human sera from dengue-1 and dengue-2 outbreaks, started in Rio de Janeiro by 1986 and 1990 respectively, were examined by electron microscopy at different times post the infection of cell cultures. More information was obtained about cell penetration of virus particles in the presence or not of antibodies, their pathway inside the cells, replication mode and exit. Infectiveness of the virus at those different stages can only be attributed to the particles appearing inside the trans-Golgi vesicles; most of all newly formed virus particles remain inside the RER-derived cell vesicles or inside lysosomes, even during cell lysis. Groups of larger particles, 65-75 nm in diameter at dengue-2 infections, persist during cell passage. The large amounts of smooth membrane structures, as vesicles or tubules inside the RER, are attributed to a cell response to viral infection.

Key words: Flaviviridae – dengue-1 – dengue-2 – virus-replication – ultrastructure

Many efforts have recently been made to elucidate the replication mode of Flaviviruses (Hase et al., 1989b) and particularly to localize structural and non-structural viral proteins inside and outside infected cells (Ng & Corner, 1989). The understanding of the endocytic pathway of particles in the cells (Gruenberg et al., 1989; Griffiths et al., 1990), of the penetration of dengue virus particles into cells (Hase et al., 1989a; Barth, 1991b, c) and of their uncoating mechanism inside the cytoplasm, contributed to elucidate Flavivirus replication. A general survey was made by Monath (1990) and Schlesinger & Schlesinger (1990).

A dengue virus type 1 (dengue-1) outbreak, started in Rio de Janeiro by March 1986 (Schatzmayr et al., 1986), was followed by an outbreak of dengue virus type 2 (dengue-2) by April 1990 (Nogueira et al., 1990, 1991). The human sera were screened by immunofluorescence and ELISA tests (Nogueira et al., 1988).

Ultrastructural observations of these viruses inside mosquito cells in culture were recently made by Barth (1991a) and Barth & Schatzmayr (1992). The appearance of morphologically distinct virus particles in dengue-2 isolates (Barth & Côrtes, 1991) and the active role of dengue-1 antibodies in infection enhancement during a subsequent infection with dengue-2 virus (Halstead, 1981; Barth, 1991b) prompted us to a more detailed study of these aspects by electron microscopy.

MATERIAL AND METHODS

Cells – The C6/36 *Aedes albopictus* cell line was mantained at our laboratory for all the experiments using L-15 medium, supplemented with 2% fetal bovine serum, 1% non-essential aminoacids and 10% tryptose phosphate broth.

Viruses – 50 µl of human serum obtained from patients previously shown as positive for Den-1 (15 samples) and Den-2 (20 samples), using methods already described (Schatzmayr et al., 1986; Nogueira et al., 1990), were diluted 1:10 with L-15 medium and inoculated separately in the C6/36 cell strain and incubated at 28 °C.

Cell/virus interactions – Den-1 and Den-2 inoculated cells were quickly washed with 7% saccharose containing phosphate buffer and fixed after 30 min, 24 hr and between three to five days according to the appearance of cytopathic effect in cell cultures. When 30 sec

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postinfection experiments were carried out, no buffer washing was applied and the fixative was directly put onto the infected cells. No previous virus adsorption at 4 °C for synchronization of virus entry into cells was performed, as it was our intention to develop a less artificial assay.

In another experiment, pools of human sera positive for Den-2 virus, were mixed with an equal volume of antibody-containing Den-1 serum, incubated at 37 °C for 30 min and fixed 30 sec and 30 min after inoculation in the C6/36 cell strain, also fixed at the appearance of 30% cytopathic effect (in general at the third day postinoculaion), and processed for electron microscopy.

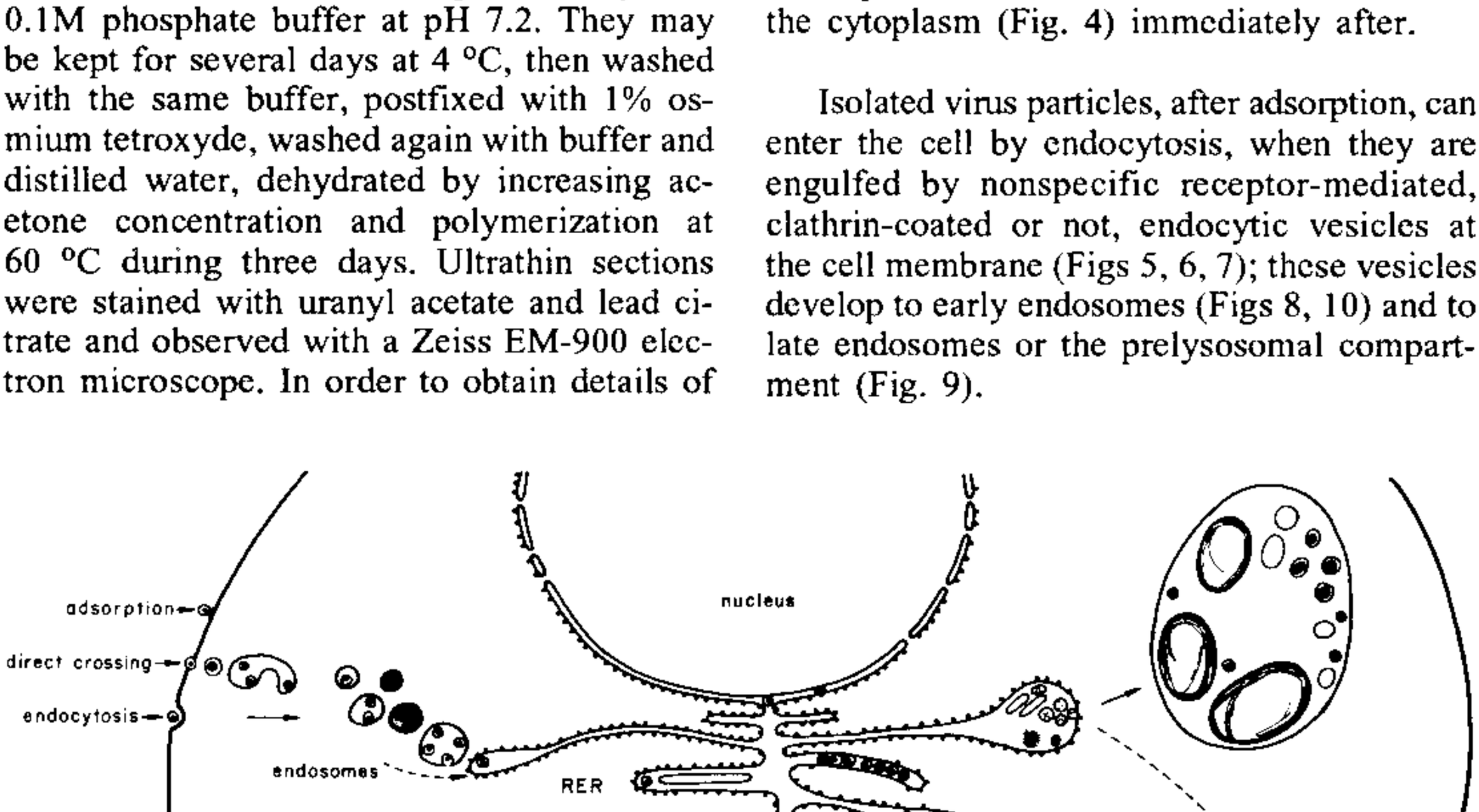
Electron microscopy – Dengue viruses infected cells were washed once with buffer (PBS) and then fixed in 1% glutaraldehyde in 0.1M phosphate buffer at pH 7.2. They may be kept for several days at 4 °C, then washed with the same buffer, postfixed with 1% osmium tetroxyde, washed again with buffer and distilled water, dehydrated by increasing acetone concentration and polymerization at 60 °C during three days. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM-900 electron microscope. In order to obtain details of viral morphology, infected cell lysates, after centrifugation, were negative stained by 2% phosphotungstic acid (PTA) in PBS, pH 7.2.

RESULTS

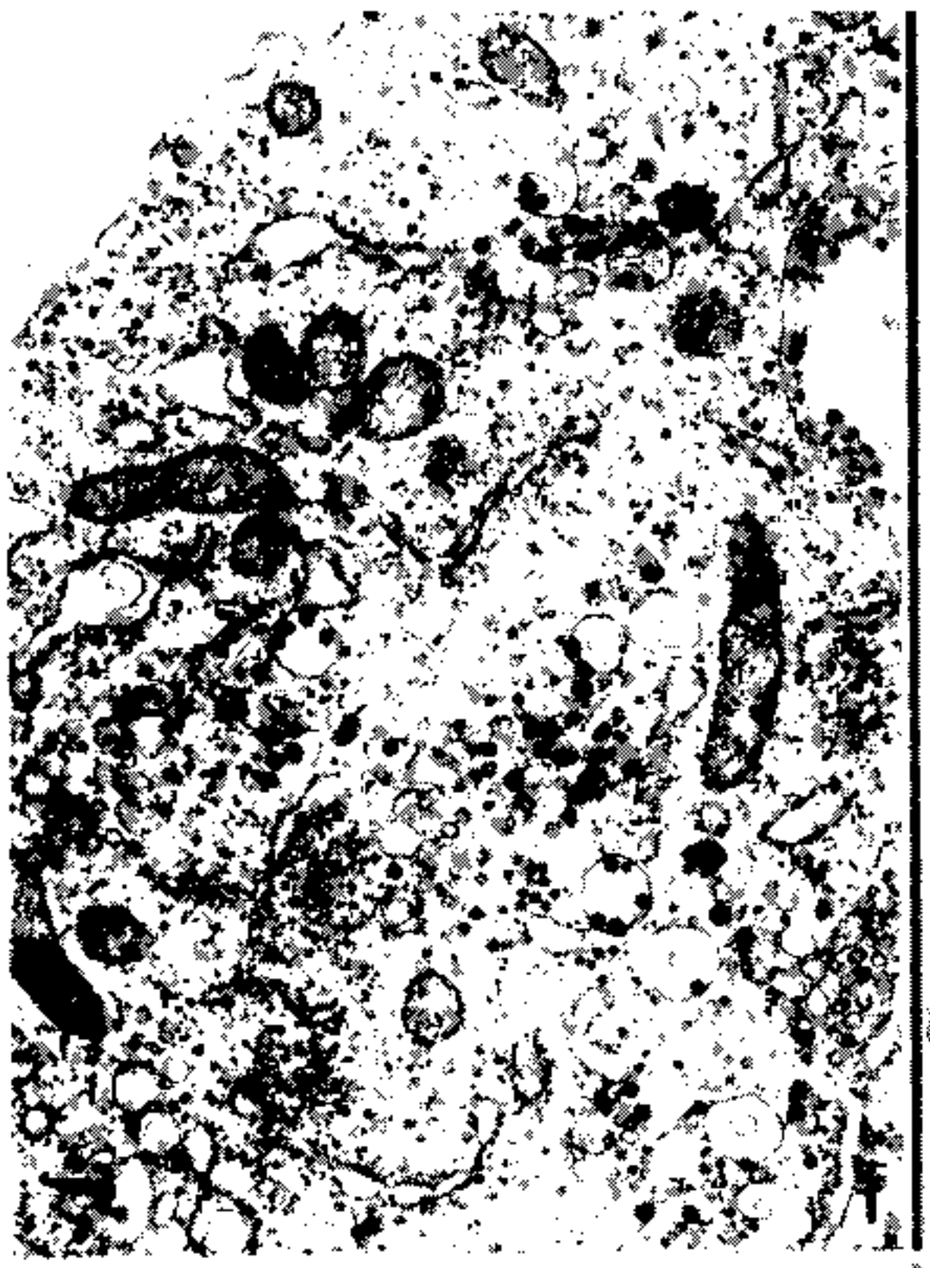
Morphological aspects of dengue viruses adsorption to C6/36 mosquito cells *in vitro*, viral entry and exit, as well as the replication mode and virus inactivation are presented as a synopsis in the schema.

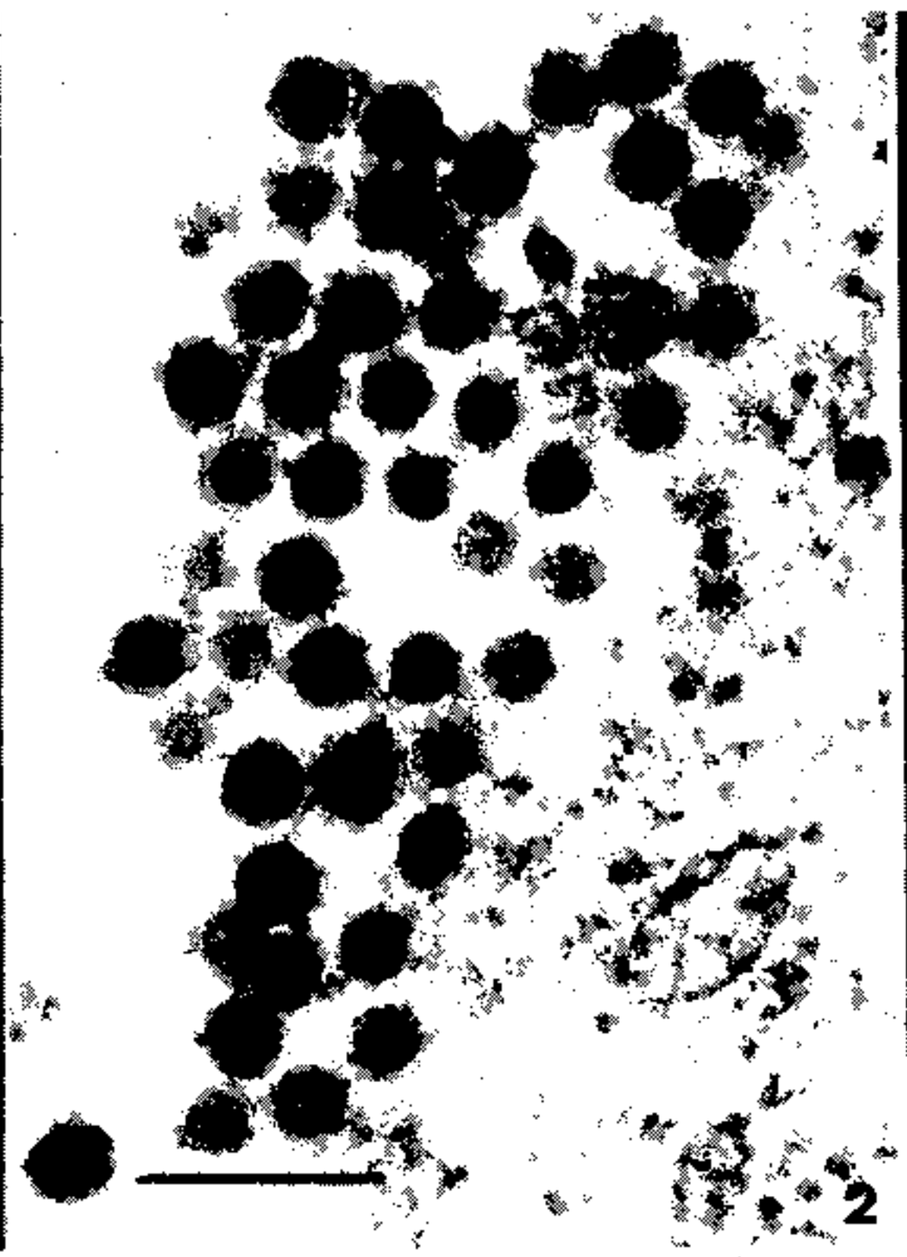
Virus entry – After 30 sec, virus particles were observed at different distances from the plasmalemma in Den-2 infected cells (Fig. 1). Some lay outside, some were adsorbed to the cell coat, groups of them might be agglutinated by antidodies if pools of sera were used (Fig. 2). They might be also found inside the plasmalemma (Fig. 3) during direct cell membrane penetration, and can be observed free in the cytoplasm (Fig. 4) immediately after.

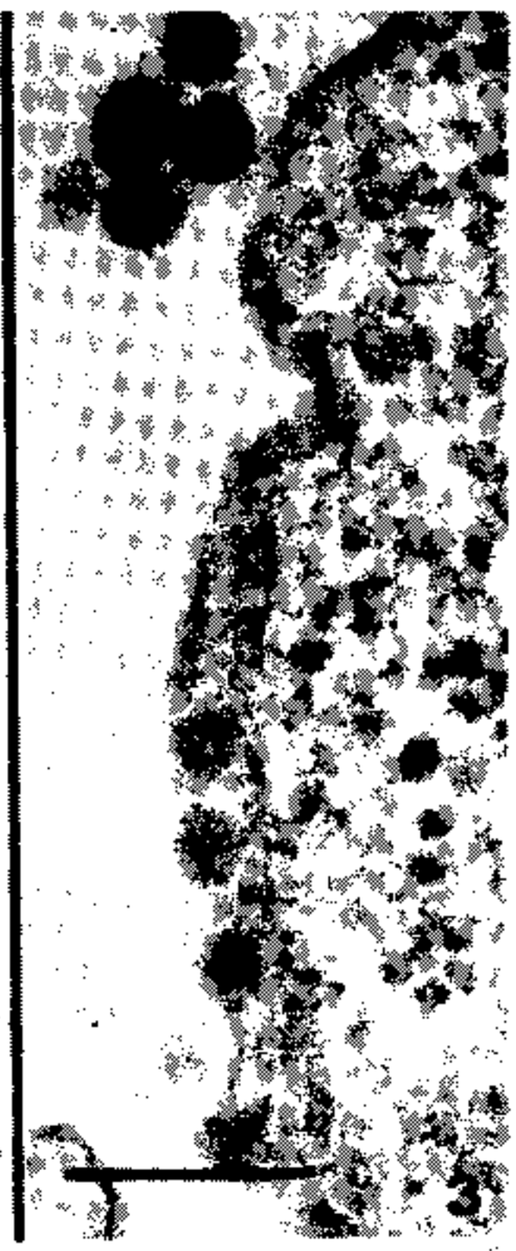
Isolated virus particles, after adsorption, can enter the cell by endocytosis, when they are engulfed by nonspecific receptor-mediated, clathrin-coated or not, endocytic vesicles at the cell membrane (Figs 5, 6, 7); these vesicles develop to early endosomes (Figs 8, 10) and to late endosomes or the prelysosomal compartment (Fig. 9).

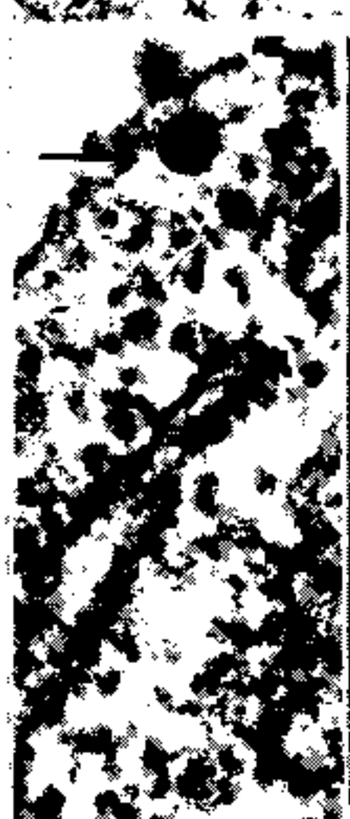


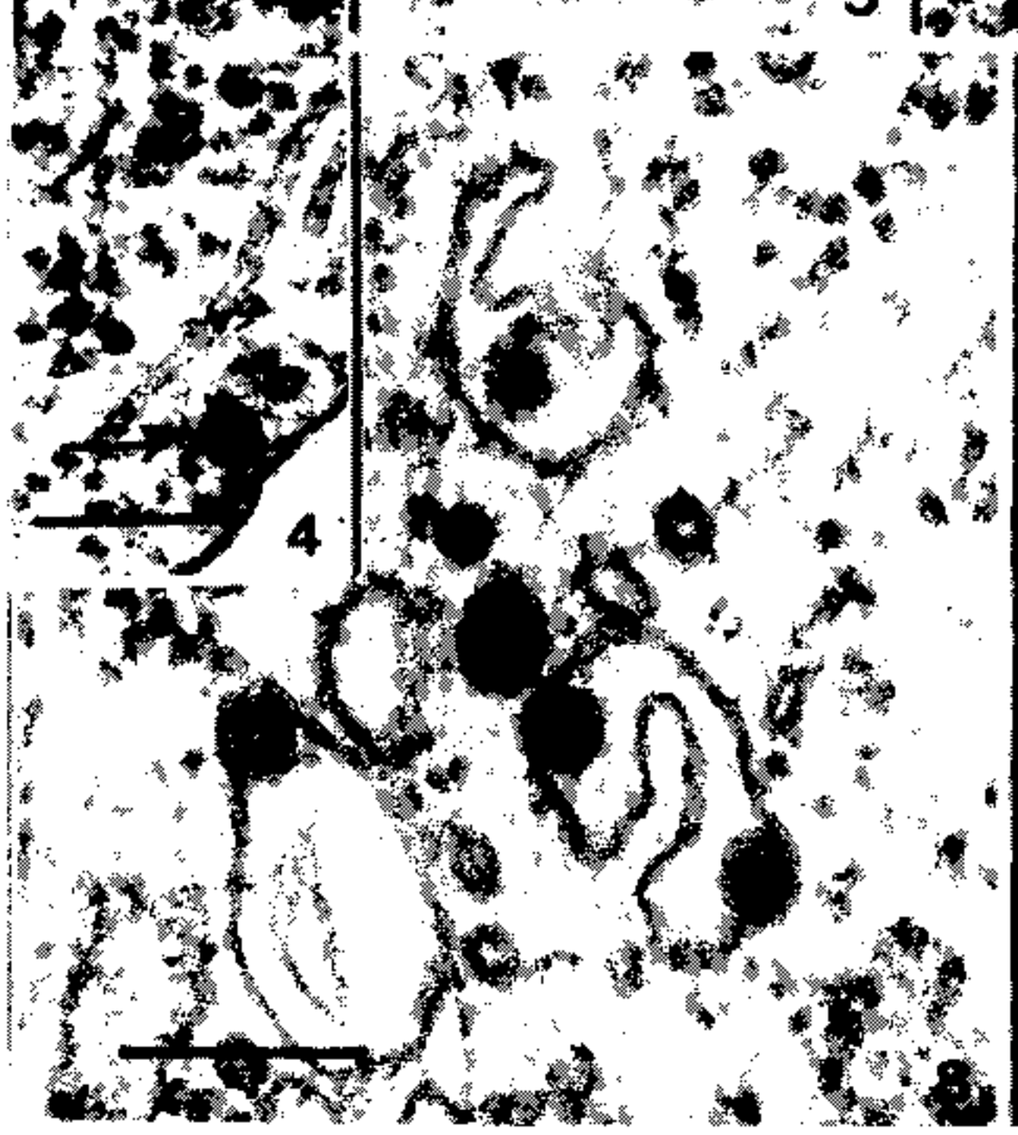
Dengue-2 virus replication in mosquito cell cultures.











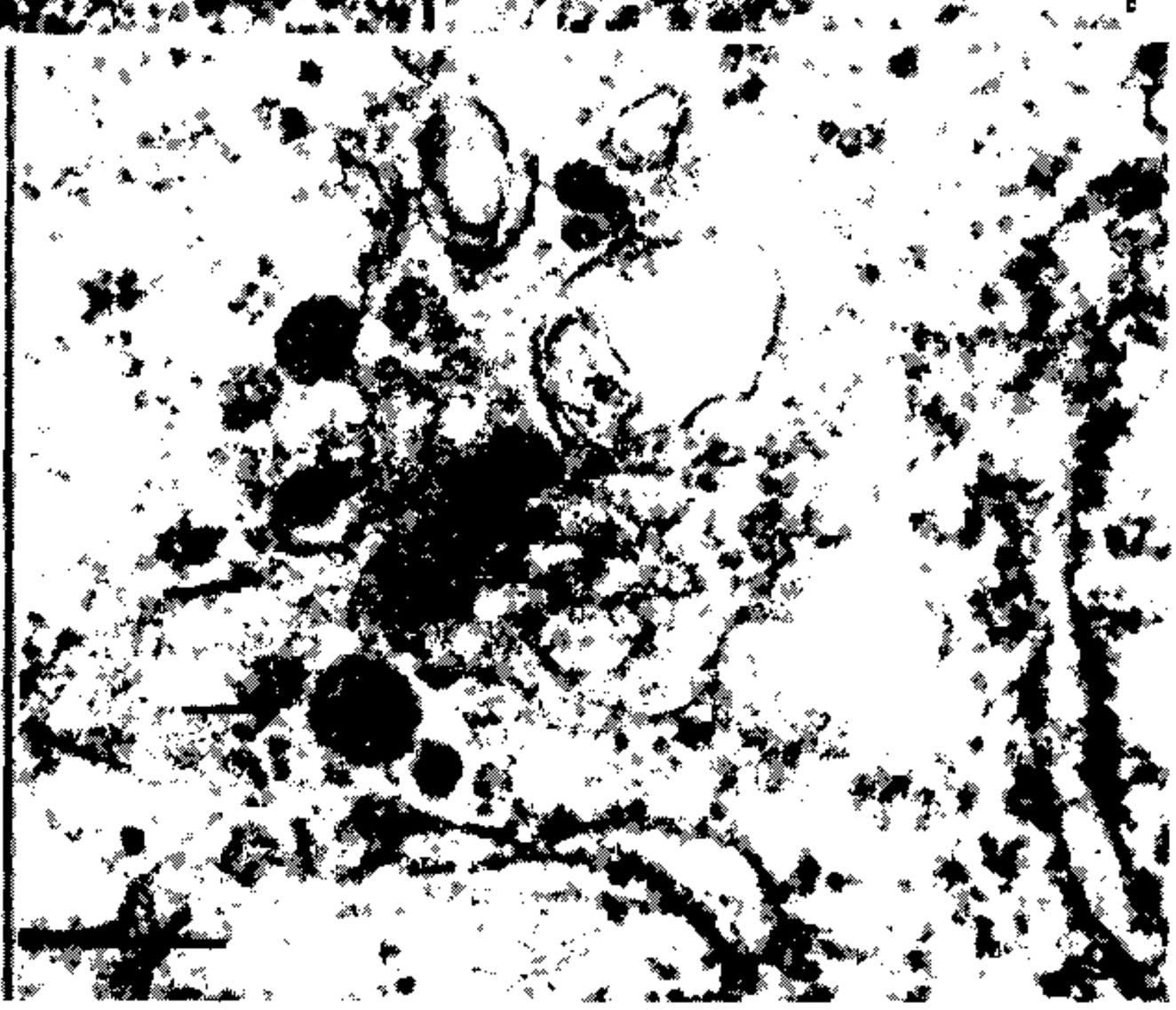
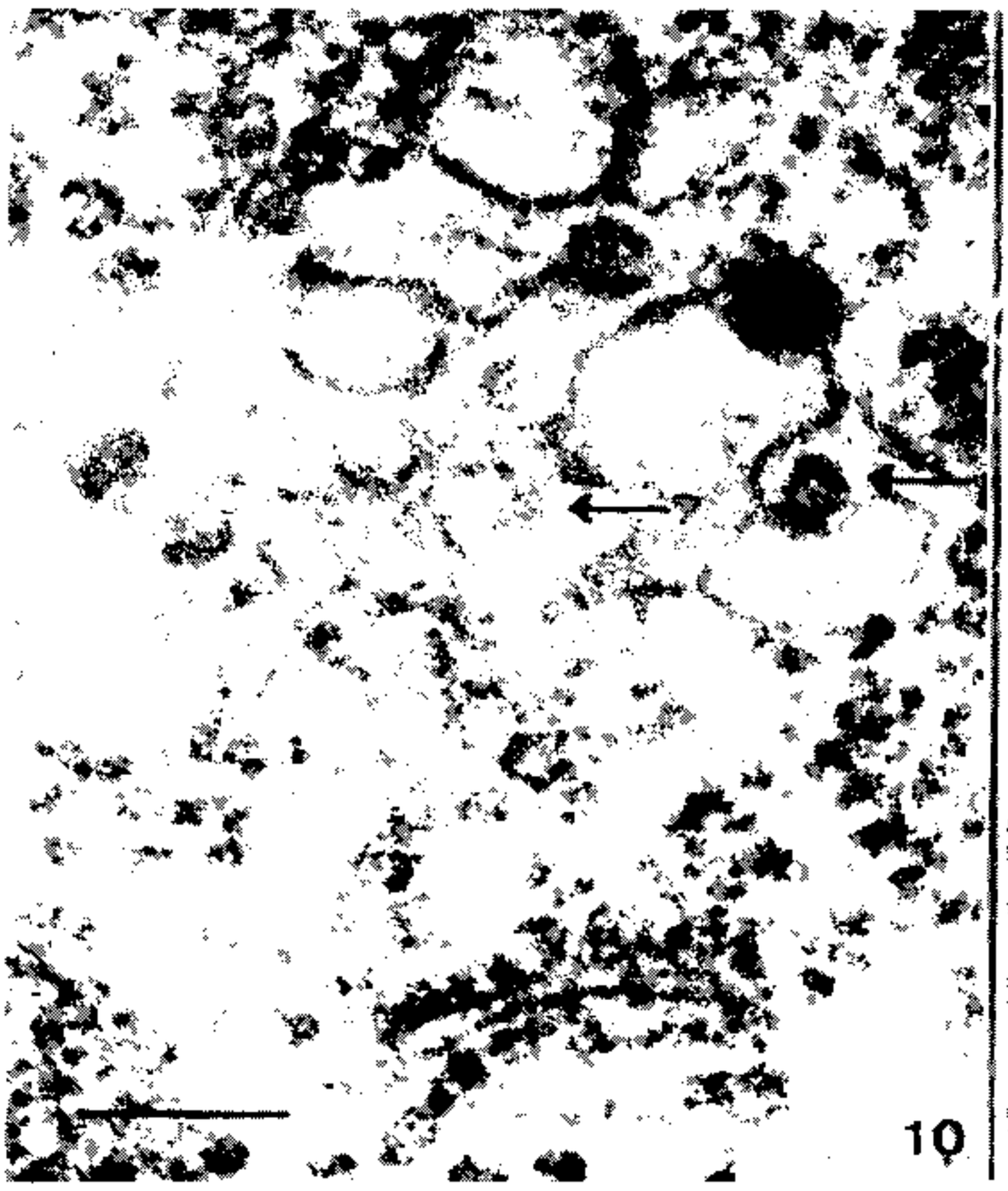
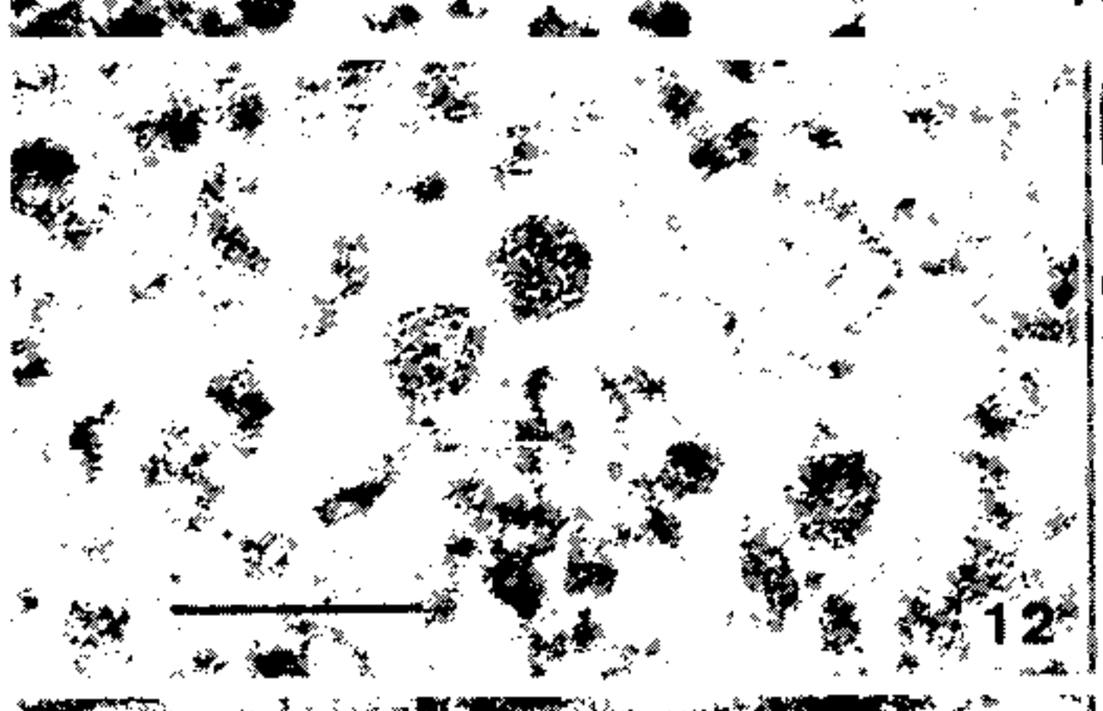
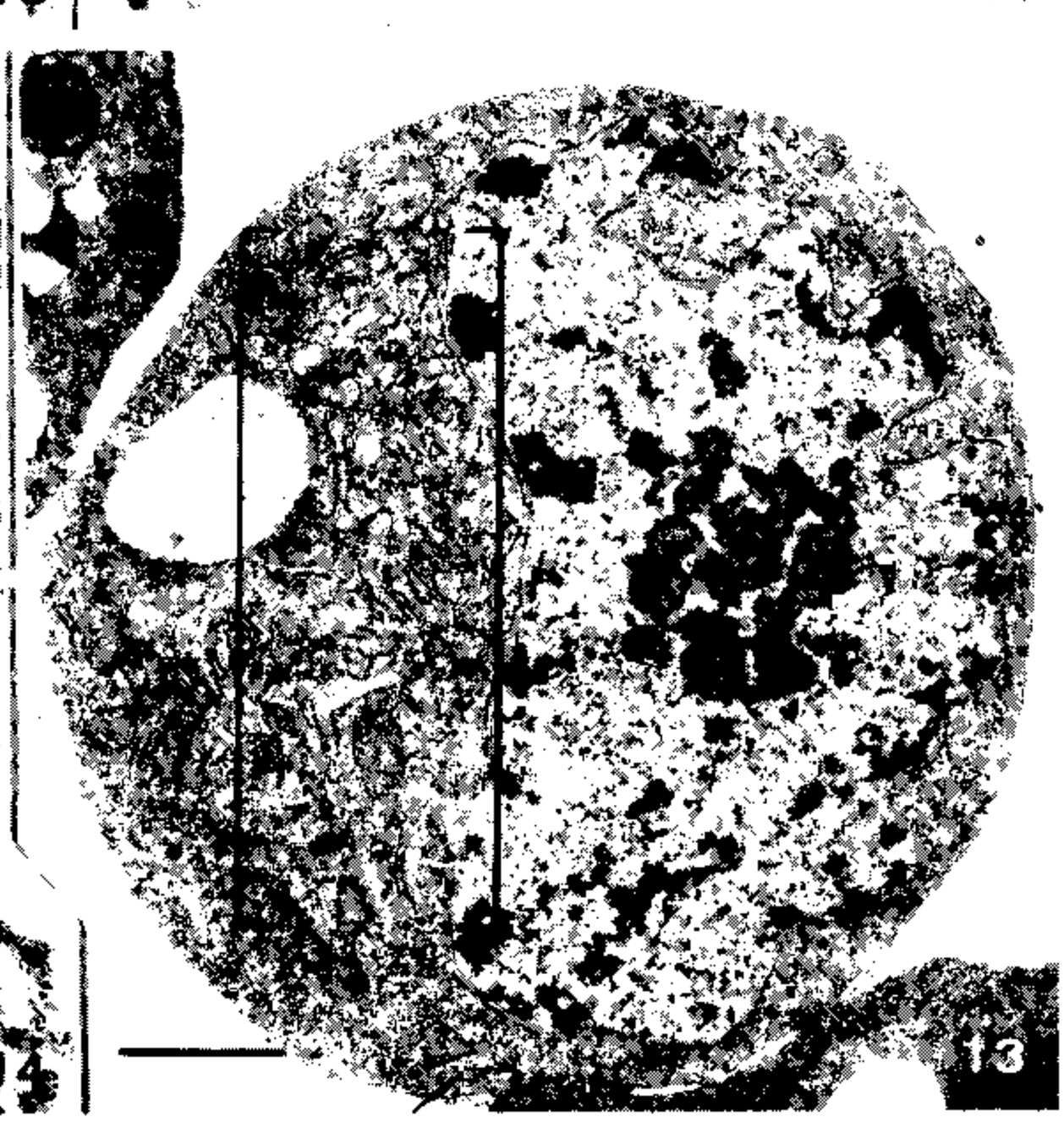


Fig. 1: mosquito cell infected by a pool of dengue positive sera, 30 sec post infection, 18000x. Fig. 2: virus particles adsorbed to the cell membrane, 75000x. Fig. 3: virus particles outside and inside the plasmalemma (arrows), 75000x. Fig. 4: two virus particles free inside the cytoplasm, 30 sec post infection, 60000x. Figs 5-7: endocytosis; Fig. 5: virus particle outside the plasmalemma and one inside a coated pit, 60000x; Fig. 6: virus particle inside an early endocytic vesicle (arrow), 60000x; Fig. 7: virus particle inside an endocytic vesicle (arrow), 60000x. Fig. 8: early endosome with virus particles, 75000x. Fig. 9: virus particle inside an endocytic vesicle (arrow) and an early endosome above, 45000x (Bars = 200nm).







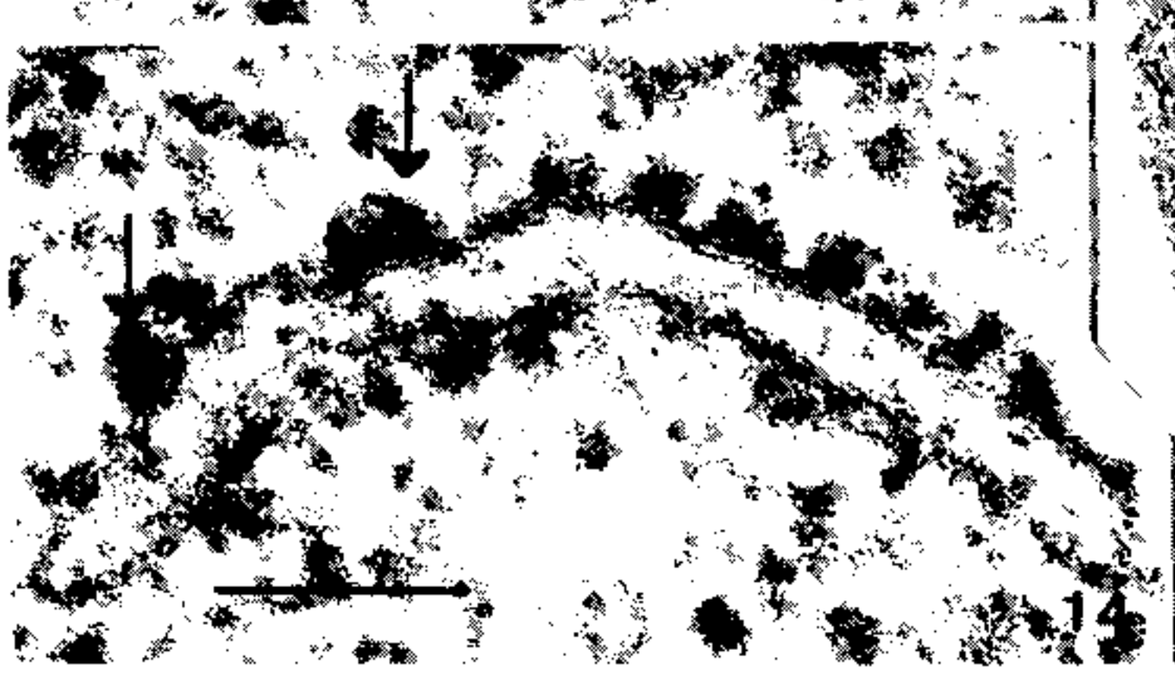


Fig. 10: virus particles inside an early endosome and free cores in the cytoplasm (arrows), 75000x. Fig. 11: numerous virus particles inside a phagosome, 30000x. Fig. 12: free viral cores inside the cytoplasm at the late endosomal or prelysosomal stage of virus cell penetration, 150000x. Fig. 13: typical structured mosquito cell 24 hr postinfection; viral replication begins inside the RER ([ ]), 12300x. Fig. 14: possible virus precursors at the cytoplasmic side of the RER (arrows), 150000x (Bars = 200 nm except Figs 12 and 14, bar = 100 nm, and Fig. 13, bar = 1 μm).

Antibody agglutinated groups of virus particles, adsorbed to the plasma membrane, enter the cell by phagocytosis, so that large phagosomes, full of a dense matrix and containing numerous virus particles, may soon be found inside the cell (Fig. 11).

These three ways of virus entry into mosquito cells occur simultaneously. In Den-1 or Den-2 infections, isolated particles were found adsorbed to or penetrating the cell membrane directly or by endocytosis, while phagocytosis was not observed.

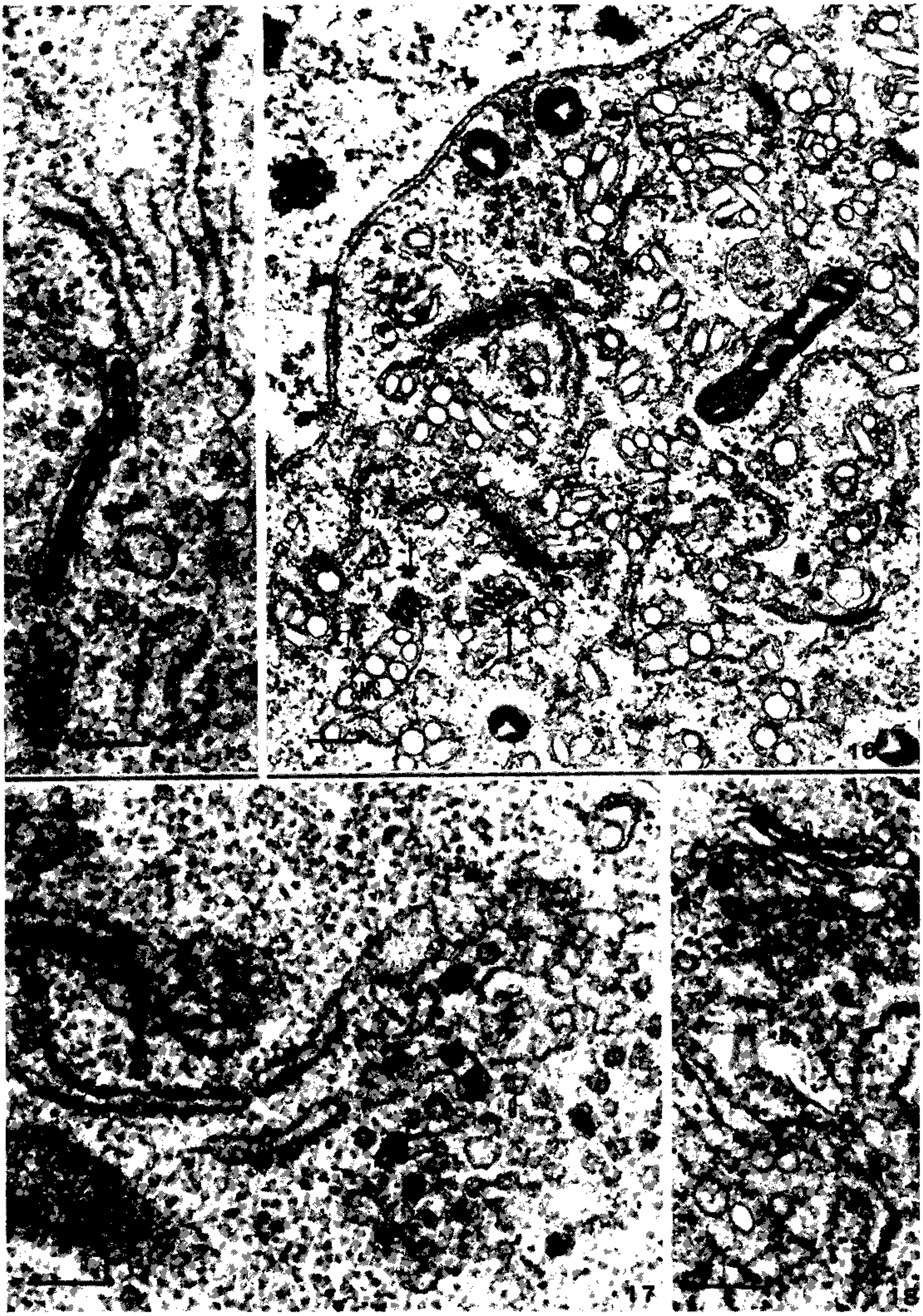
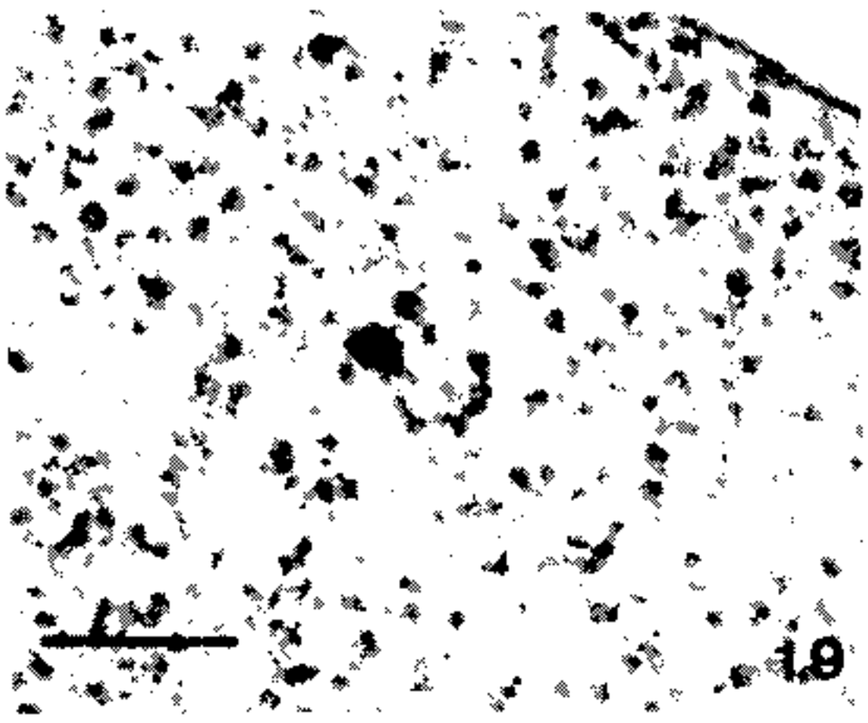
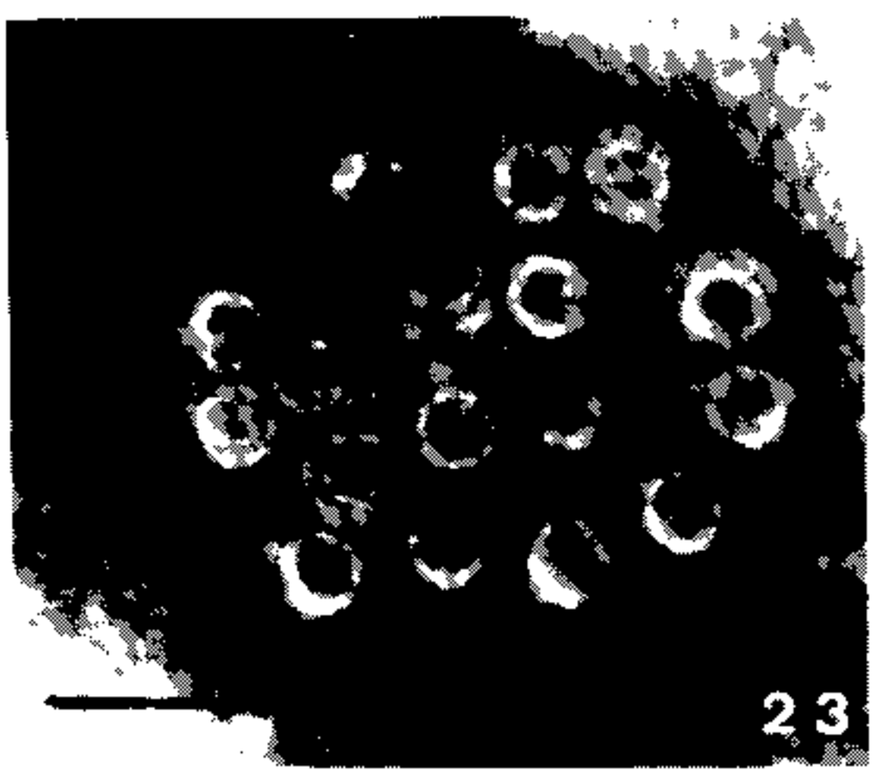


Fig. 15: newly formed virus particles inside the endoplasmic reticulum, where no more ribosomes are present at the cytoplasmic sides of the RER, 60000x. Fig. 16: virus particles (arrows) and smooth membrane structures (SMS) inside rough and smooth membrane bound vesicles, 40000x. Figs 17 and 18: virus particles are tranferred by vesicles to the Golgi complex (G), 60000x (Bars = 200 nm).





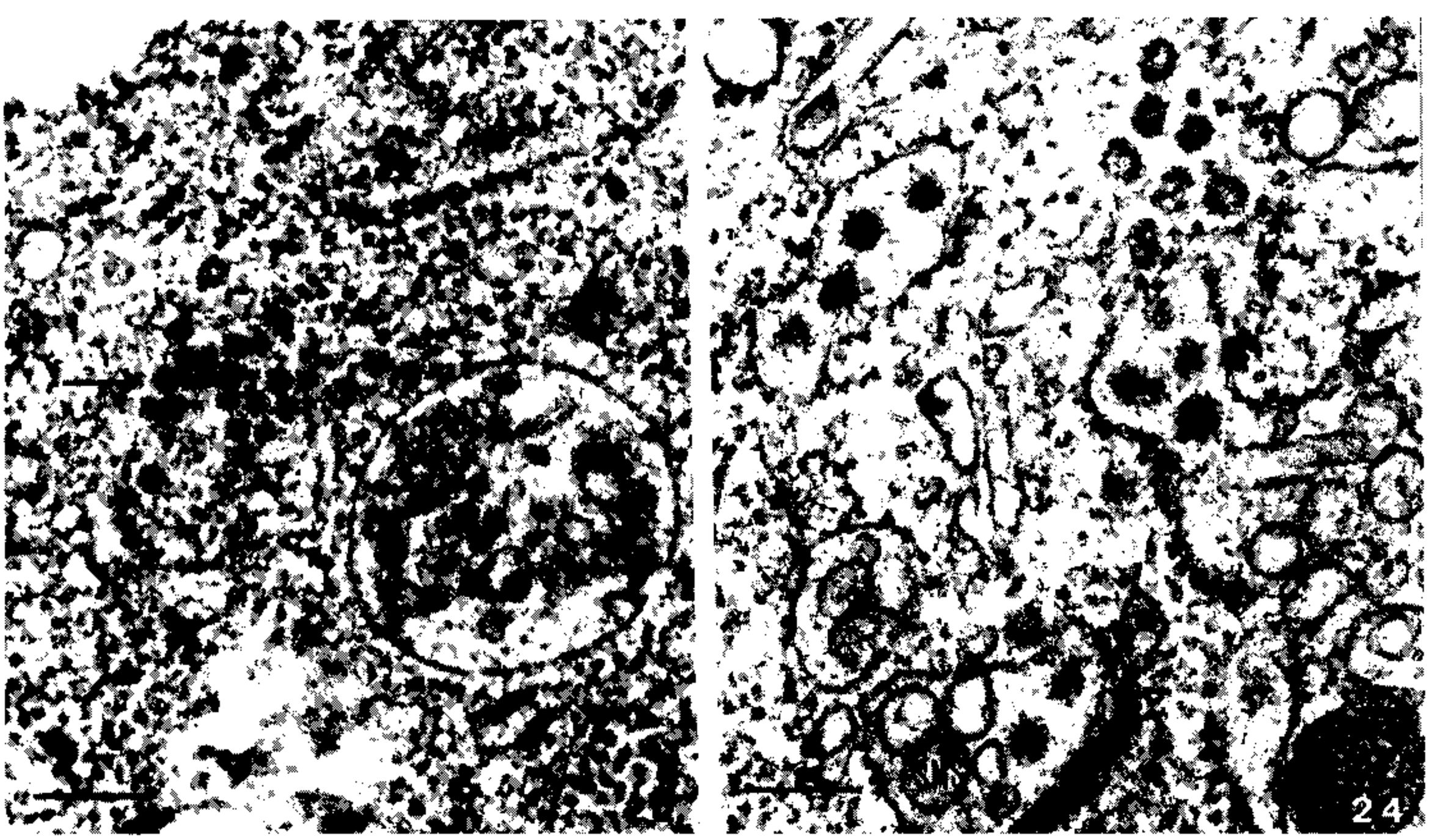


Fig. 19: single virus particle free in the cytoplasm after the replication stage, 60000x. Figs 20 and 23: negative stained virus particles inside smooth membrane bound cell vesicles; note the granular aspect of the virus surfaces at Fig. 23, 52500 and 155000x. Fig. 21: smooth membrane coated vesicles with numerous virus particles inside the cytoplasm, 14000x. Fig. 22: lysosome with virus particles; another group in a smooth membrane bound vesicle (arrow), 60000x. Fig. 24: SMS and fuzzy coated virus particles inside the RER in a dengue-2 infected mosquito cell, 60000x (Bar = 200 nm except Fig. 21, bar = 1 μm, and Fig. 23, bar = 100 nm).

Virus transport – After 30 min of infection, virus particles are always found in cell vesicles and tubules like early and late endosomes. Each transport vesicle contains one or more viral particles and an electron-translucent matrix, being later transferred to prelysosomal compartments and to cell lysosomes, or uncoated for virus replication (Figs

Fig. 25: usual and fuzzy coated virus particles and SMS inside a same RER vesicle (arrow); note the numerous SMS surrounded by smooth and rough membranes, 60000x (Bar = 200 nm).

10, 12). The dense matrix of phagosomes containing virus particles may be related to antibody digestion, since it is never observed inside cell lysosomes.

Later, 24 hr postinfection, no virus particles are found inside cell endosomes; only within lysosomes they are found in a kind of advanced degrees of digestion. At this stage, viral replication is initiated and no morphological virus-related structures may be detected.

Virus replication – From the second to the third day postinfection, beginning after 24 hr (Fig. 13), possible virion precursors are found on the cytoplasmic sides of the rough endoplasmic reticulum (RER) (Fig. 14). Increase of ribosome size and density suggests synthesis of viral proteins and binding of viral RNA. A very quick introduction of these complexes into the RER cisternae provides evidence that viral particles are formed involved by a proteinous membrane directly inside the RER cisternae.

During the most active phase of virus multiplication, after penetration into the RER cisternae, most ribosomes are no longer found on this side of the RER membranes, which are now transformed into smooth membrane-coated, viruses-containing cell vesicles (Figs 15, 16).

Virus maturation and cell release – Once inside the RER, only very few virus particles are transferred by vesicles to the Golgi complex. They are found inside smooth or clathrin-coated vesicles (Figs 17, 18), developing probably to infective viral particles, to be transported inside these vesicles to the plasma membrane. Fusion of the plasmalemma with the vesicle membrane release the virus particle from the cell by exocytosis.

Some viral particles are found free inside the cytoplasm, next to microfilaments directed towards the cell membrane (Fig. 19). Maybe they can pass the cell membrane as they can enter the cell.

Most newly formed virus particles remain isolated or grouped inside cell vesicles derived from the RER cisternae (Figs 20, 21, 23), and inside the lysosomes (Fig. 22); they never leave the cell. Fusion of those vesicles and lysosomes with the plasmalemma was never observed.

Smooth membrane structures (SMS) – These structures are rounded vesicles, shorter or longer tubules, whose diameter changes between 70 and 100 nm; they occur inside the cell RER during virus replication (Figs 16, 25). They first appear when virion synthesis is beginning at the cytoplasmic sites of the RER, about 24 hr after cell infection.

SMS bud from the membrane inside the RER as it can sometimes be observed at ultrastructural level. They appear before new virus particles are detected inside the RER and indicate the sites of the RER cysternae where viral assembly is beginning.

Den-2 viruses – In all Den-2 isolates examined by electron microscopy, a second type of viral particles is always present inside the RER. Their cores are equal or a little larger than the common viral particles, but the envelope differs. Dengue virus envelope, due to the E proteins, appear as membranes with a finely granular aspect, as observed by negative staining (Fig. 23) and in ultrathin section (Barth, 1991 a, b, c). The different viral particles detected in Den-2 infected C6/36 cells have a larger envelope with longer projections, looking like a fuzzy coat (Figs 24, 25). Their diameters change between 65 and 75 nm. They occur isolated or in groups inside the RER cisternae and can even be detected in lysed cells, inside vesicles or lysosomes. They are present even at the fifth cells passage, occurring together with the usual dengue virus particles (Fig. 25).

DISCUSSION

Replication mechanism of flaviviruses is not completely understood (Schlesinger & Schlesinger, 1990). Information obtained by several authors, (Ng & Corner, 1989; Hase et al., 1989b) bring out details that would suggest the mechanism of flavivirus replication. Except for one Den-2 strain (Hase et al., 1989b), no similarities with alphavirus replication (Hase et al., 1989b; Mesêncio et al., 1989; Schlesinger & Schlesinger, 1990) are observed, where budding is the only known way of viral replication and exiting the cell.

Virus adsorption is enhanced by antibodies and Fc receptors present at the cell membrane (Schlesinger & Schlesinger, 1990). These antibodies should not be virus-type specific, since large amounts of virus particles are found adsorbed to the mosquito cell membranes in the presence of Den-2 viruses and Den-1 antibodies.

Large groups of virus-antibody complexes are phagocytosed by the cell, and antibodies are denaturated inside the phagosomes at a lower pH. In our observations, virus particles are never released from the phagosomes into the cytoplasm. The dense matrix becomes more electron-translucent during its development, perhaps by addition of Golgi vesicle substances,

and the virus particles lose their proteinous envelope. Upon cell lysis, they can remain as more or less dense bodies or, frequently, they develop into cell lysosomes. No replicating function can be attributed to the phagocytic pathway of virus particles inside the cells.

The endocytic pathway of virus entry into mosquito cells may be the same for alpha- and flaviviruses (Schlesinger & Schlesinger, 1990), but, differently than is observed in some alphaviruses, fusion of flavivirus particle envelope with the plasma membrane was never observed. In the endosomes, uncoating of virus particles is a very quick process (Gruenberg et al., 1989; Griffiths et al., 1990), rarely observed by electron microscopy (Figs 10, 12), as viral cores are immediately dissociated inside the cytoplasm; the lack of any morphologically complete viral particles or viral cores free inside the cytoplasm at this time of infection (latent period from 6 to 18 hr according to Hase et al., 1989b), was previously suggested by Westaway (1980).

During the latent period of infection, virion single-stranded RNA is translated by minus RNA strands into plus strands inside the cytoplasm and viral protein synthesis is initiated (Schlesinger & Schlesinger, 1990). The first detectable structured newly formed virion cores, linked to RER ribosomes, seem to be involved by the core protein (Fig. 14) and, passing the RER membrane, to receive the envelope. As the major part of these virions never leave the RER, only a few are transferred to Golgi vesicles (Figs 17, 18), where glycosylation of its envelope proteins occurs (Leary & Blair, 1980). Clathrin-coated or uncoated Golgi vesicles make the transport of these virus particles to the plasma membrane, where they are released by exocytosis. Maybe that only virions passing the Golgi cysternae are infective. The ratio between noninfective and infective virions has yet to be established. By electron microscope observation of several Den-1 and Den-2 infections, this ratio seems to be higher with Den-2 strains while, enhanced or not by antibodies, a larger amount of virions are synthesized inside the RER cysternae (Barth, 1991a). Quicker and more fulminant clinical symptomes may be attributed to this fact.

During viral replication, no morphologic relationship between the virus particles containing RER and the ER (smooth endoplasmic reticulum) can be observed, since a relation between the ER and viral protein glycosylation was hyppothesized by Stohlman et al. (1975). On the other hand, budding from cell membranes as in alphavirus infection (Mezêncio et al., 1989) was never observed.

Like in the Den-1 and Den-2 virus strains previously examined (Barth, 1991a, Barth & Schatzmayr, 1992) and other flaviviruses (Ko et al., 1979; Leary & Blair, 1980), SMS occur early in mosquito cells infected by dengue viruses. Its mode of appearance inside the RER (Barth, 1991a; Hase et al., 1987b) or inside the cytoplasm (Ng & Hong, 1989) depends upon the infected cell origin, from insects or mammals. Several cytochemical tests (Ng & Corner, 1989; Barth & Schatzmayr, 1992), add no information about its nature. In this respect we now consider that these structures represent a response of the cell, induced by viral infections (Staeheli, 1990), and that the SMS contents maybe proteins like antiviral substances.

In mosquito cell infections by Den-2, two types of virus particles were simultaneously found (Barth, 1991a). One, like other dengue viruses, is 37-50 nm in diameter (Schlesinger & Schlesinger, 1990), while the other is larger. In order to check out a possible antibody origin of its fuzzy coat, a dengue virus pool (Den-1 + Den-2), previously incubated at 37 °C, was inoculated into mosquito cells and observed up to the fifth passage, with the result that the larger virus particles were always present inside the cell RER together with the usual ones. Those unusual virus particles have also been detected in other flavivirus infections (Prof. Dr F. Murphy, personal communication), but their significance remains obscure.

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