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BRAZILIAN DENGUE VIRUS TYPE 1 REPLICATION IN MOSQUITO CELL CULTURES

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The development of dengue viruses type 1 obtained from accute human sera and inoculated into mosquito cell cultures, was observed by standard transmission electron microscopy and cytochemical staining. It follows the trans-type mechanism already established of other dengue types. Direct passage of single virus particles across the cell membrane seems to be a pathway of entry and exit in dengue-1 infected cells. The nature of numerous electron translucent vesicles and tubules, produced simultaneously during virus replication inside the rough endoplasmic reticulum, was analyzed by cytochemical tests. The largest amount of virus particles was produced inside cell syncytia.

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

A dengue virus outbreak started by March 1986 in Rio de Janeiro and spread very rapidly to other states of the country (Schatzmayr et al., 1986). The virus was identified as dengue type 1 (dengue-1) being isolated from human sera and vectors and its identity confirmed by immunofluorescence using monoclonal antibodies (Schatzmayr et al., 1986; Nogueira et al., 1988).

Flavivirus replication takes place in the host cell cytoplasm. Two different mechanisms of virus maturation in C6/36 cells were proposed by Hase et al. (1989b). During the cis-type maturation, viral nucleocapsids bud from cytoplasmic membranes into cell vacuoles or from the plasma membrane into the extracellular medium, as may be observed with dengue-2, PR-159 strain. When trans-type maturation occurs, virions from inside the rough endoplasmic reticulum (RER) migrate through the Golgi cisternae, leaving the cells in exocytosis vesicles (Leary & Blair, 1980). Most flaviviruses have this type of maturation, in special dengue viruses types 1, 2, 3 and 4 (Ko et al., 1979), Japanese encephalitis viruses and Kunjin viruses (Hase et al., 1989b). However, dengue-1 virus replication has been poorly studied, and most research deals with dengue-2 virus in different cell line cultures (Hase et al., 1989a; Ng & Corner, 1989).

Virus-induced vesicles, suggestive of trans-type maturation events, formed in the cytoplasm, were observed during the replication of other viruses, such as poliovirus (Bienz et al., 1983) and plant viruses (Martelli & Russo, 1984), but never inside the RER as with flaviviruses (Ko et al., 1979; Leary & Blair, 1980); they were named smooth membrane structures (SMS) by Leary & Blair (1980). Demsey et al. (1974) observed numerous multivesicular bodies after 48 hr of cell infection with dengue-2 viruses. All these types of vesicles are considered related to virus replication and are noticed after start of viral RNA replication.

Electron microscope observations of inoculated mosquito cell cultures by ultrathin sections and negative staining, showed the occurrence of relatively few virus particles inside infected cells and cell homogenates, although, at the same time, a higher infectivity titer was observed (Barth et al., 1987; Barth, 1989a, b; Barth & Schatzmayr, 1990). Here, we describe further studies on the Brazilian dengue-1 virus strain replication in cultures cells using electron microscopy and cytochemistry.

MATERIAL AND METHODS

Virus cultivation – Ten positive human sera obtained during dengue-1 outbreak in Rio de

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Janeiro were inoculated in *Aedes albopictus* C6/36 cell culture, as described by Nogueira et al. (1988). When presenting 30-50% viral cytopathic effect, after five or seven days of inoculation, the cells were processed for electron microscopy.

Electron microscopy: negative staining – Infected cell cultures were frozen twice, inactivated by the addition of 1% glutaraldehyde and pelleted for 15 min in a Spin I, Eppendorf-type centrifuge or for one hour by ultracentrifugation at 50,000 rpm. One drop (20 µl) of the resuspended sediment was applied to a formvar or collodium carbon coated grid and stained by 2% phosphotungstic acid (PTA) as described by Barth (1984).

Electron microscopy: ultrathin sections – Infected cell cultures were fixed with 1% glutaraldehyde in phosphate buffer, pH 7.2, at equal volume of cell medium and left overnight at 4 °C. The cells were scraped from the plastic vials with a rubber-policeman and transferred to Eppendorf centrifuge tubes; they were dehydrated and polymerized remaining inside these tubes just to the final step of block preparation. After the dehydration by increasing acetone concentration and in-block staining with 1% uranyl acetate in 70% acetone, the cells were embedded in a hard Epon mixture, cut with a diamond knife and stained with uranyl acetate and lead citrate.

Cytochemistry – Different methods were used: a) cerium chlorid treatment during cell fixation (Robinson & Karnovsky, 1983) for detection of acid phosphatase activity inside cell organells; b) osmification for evidence of Golgi vesicles during cell fixation (Psenicnik & Pipan, 1989); c) enhancement of membranes by the omission of OsO4 during fixation and alcoholic 2% PTA treatment during cell dehydration; d) viral RNA enhancement was carried out by Bernhard's EDTA method (Bernhard, 1969) on ultrathin sections collected on nickel grids: the uranyl acetate stained DNA structures are discolored by this treatment.

Controls comprehend the omission of the cytochemical treatment introduced in relation to the procedure described above under "ultrathin sections".

RESULTS

In order to study dengue virus replication in mosquito cells, negative staining was used to recognise virus particles in these preparations. Groups of virus particles were usually found inside the RER together with SMS (Fig. 1). Single virus particles were occasionaly detected.

Dengue-1 virus induced C6/36 cell morphology alterations – Mosquito cells infected by dengue-1 virus from patient sera, develop a large vacuolization and an increased cell volume. Simultaneously, the nucleus is displaced to the cell periphery (Fig. 2). At a more advanced stage, the formerly dense cytoplasm becomes more translucent and the cell emptier, followed by disruption of the plasma membrane and cell organelles release into the culture medium.

With our dengue-1 viruses, frequently syncytia formation is observed (Figs 3, 4). Two morphological types of syncytia may simultaneously occur in the cell cultures. One is the type often described by light microscope observations and explained by virus-induced cell fusion. The nuclei are disposed more or less concentrically, separated by vacuoles, with a virus containing central area. The other type is represented by very large elongated syncytia, where nuclei alternate with groups of vacuoles, mitochondria and virus containing RER.

Dengue-1 virus replication in C6/36 cells – Further the RER cell compartment expands, limiting the virus replication area from other cell activities (Fig. 5). It comprises a large amount of RER tubules containing virus particles; simultaneously, virus associated electron translucent smooth membrane structures, not yet well identified, occur also as round vesicles and short or long tubules inside the RER with diameter varying from 70 to 100 nm. The virus-producing RER cell compartment is always associated with the nuclear membrane where virus particles may be found inside (Figs 7, 8). A connection with the plasma membrane was never observed (Fig. 6).

At a later stage of development, some enveloped virus particles may be found free in the cell cytoplasm, near to the plasma membrane; they seem to be associated to cytoplasmic filaments, being found soon outside the cell membrane (Fig. 9). Virus-containing exocytosis vesicles are occasionally observed.

Cell debris resulting from syncytia contain numerous virus particles inside distended RER

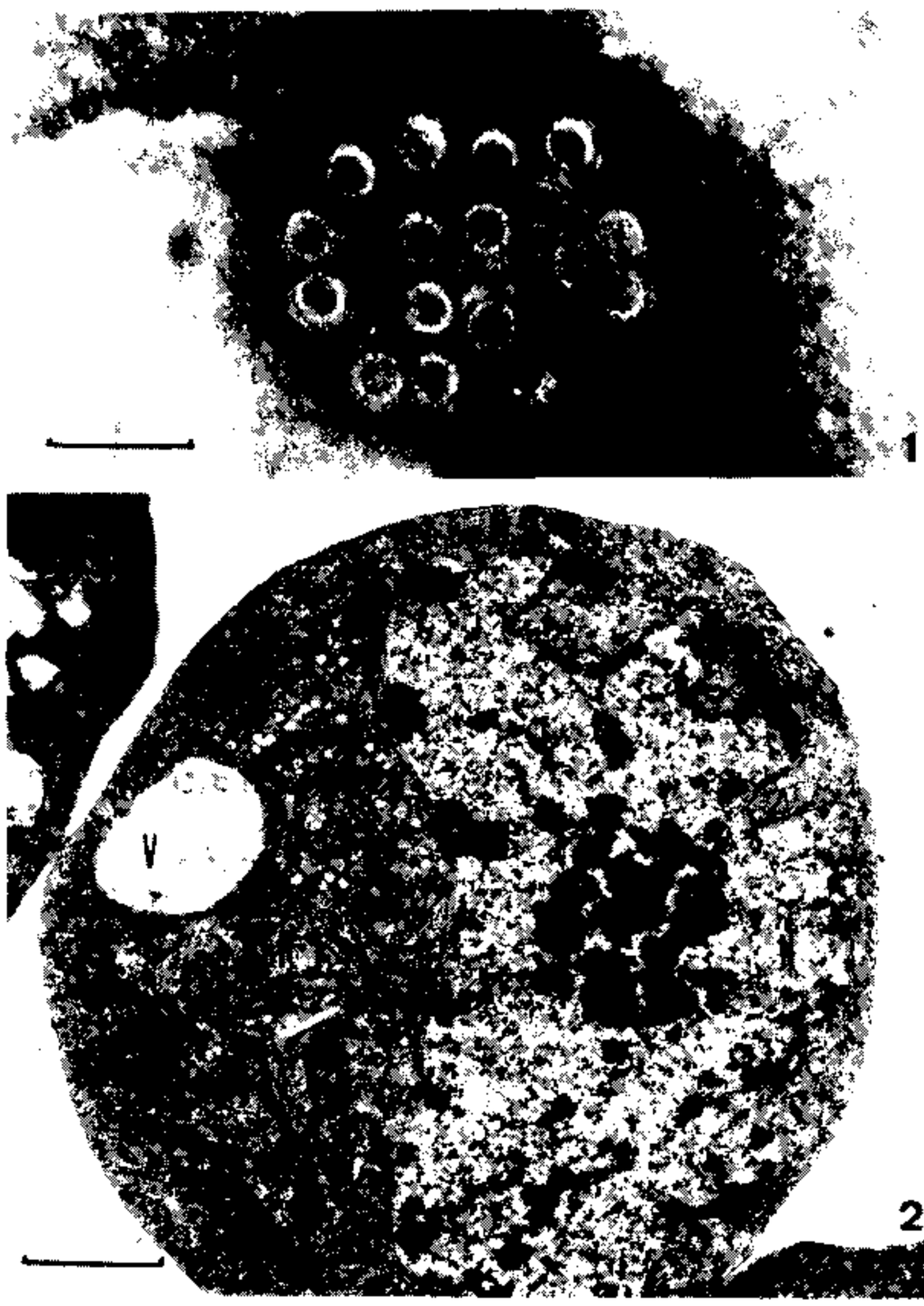
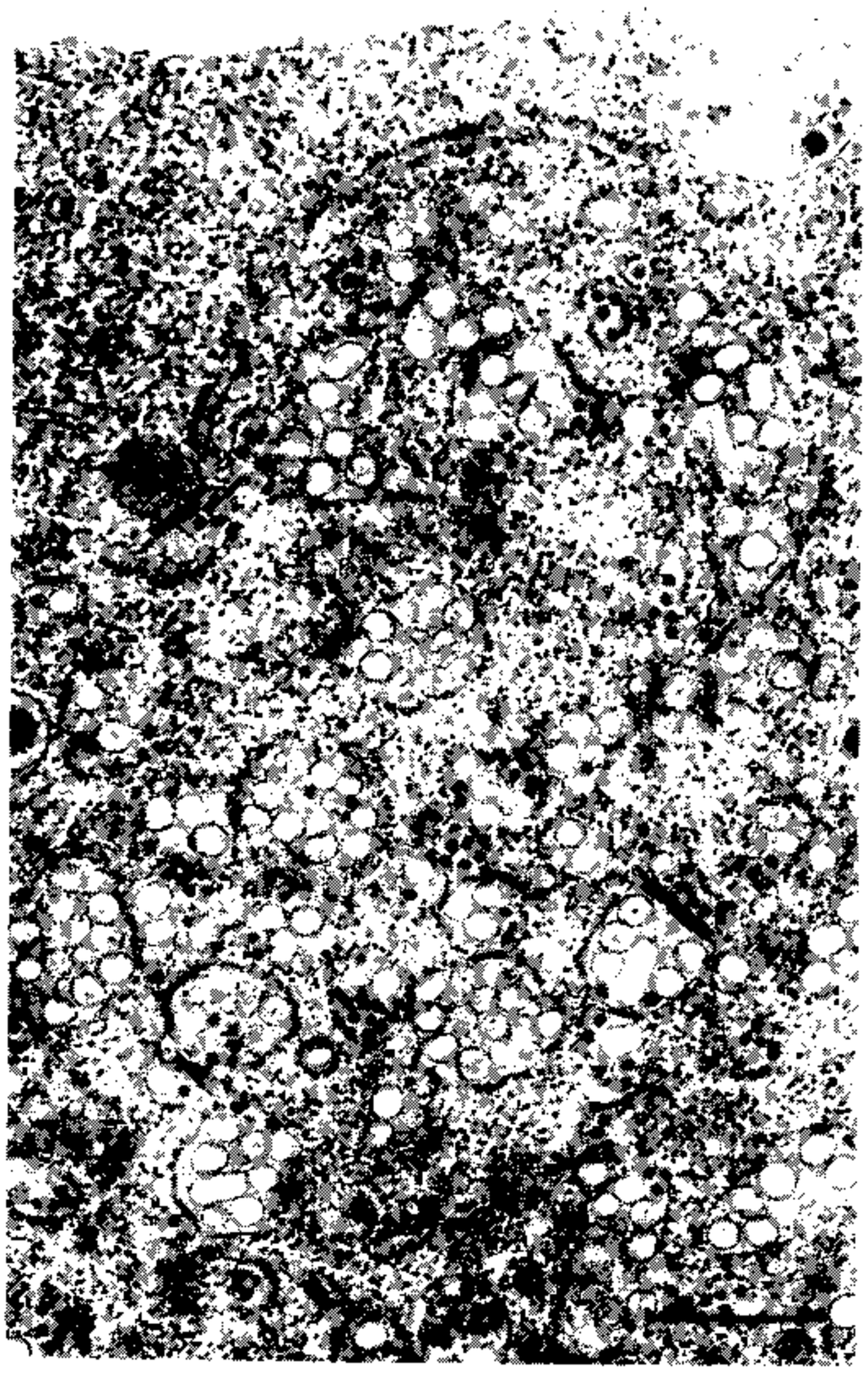
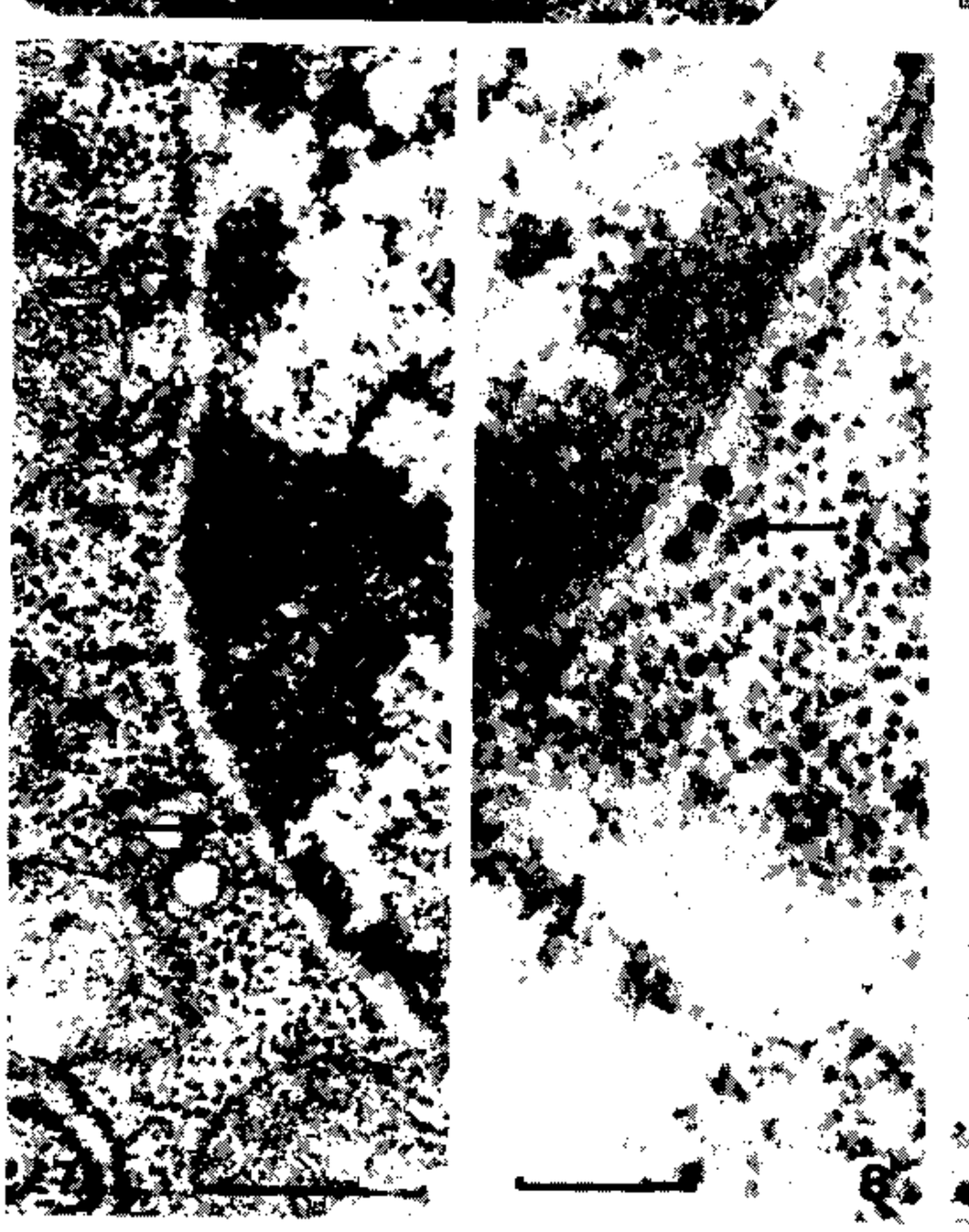


Fig. 1: group of virus particles inside a vesicle after cell lysis; negative staining, bar = 100 nm. Fig. 2: infected cell with displaced nucleus (N), virus and smooth membrane structures containing RER, vacuole (V) and mitochondria (M), bar = 1 μm. Fig. 3: elongated syncytium with one virus-producing RER region, bar = 2 μm. Fig. 4: rounded syncytium with one virus-producing RER region and acid lysosomes, stained by cerium chlorid (L), bar = 5 μm.





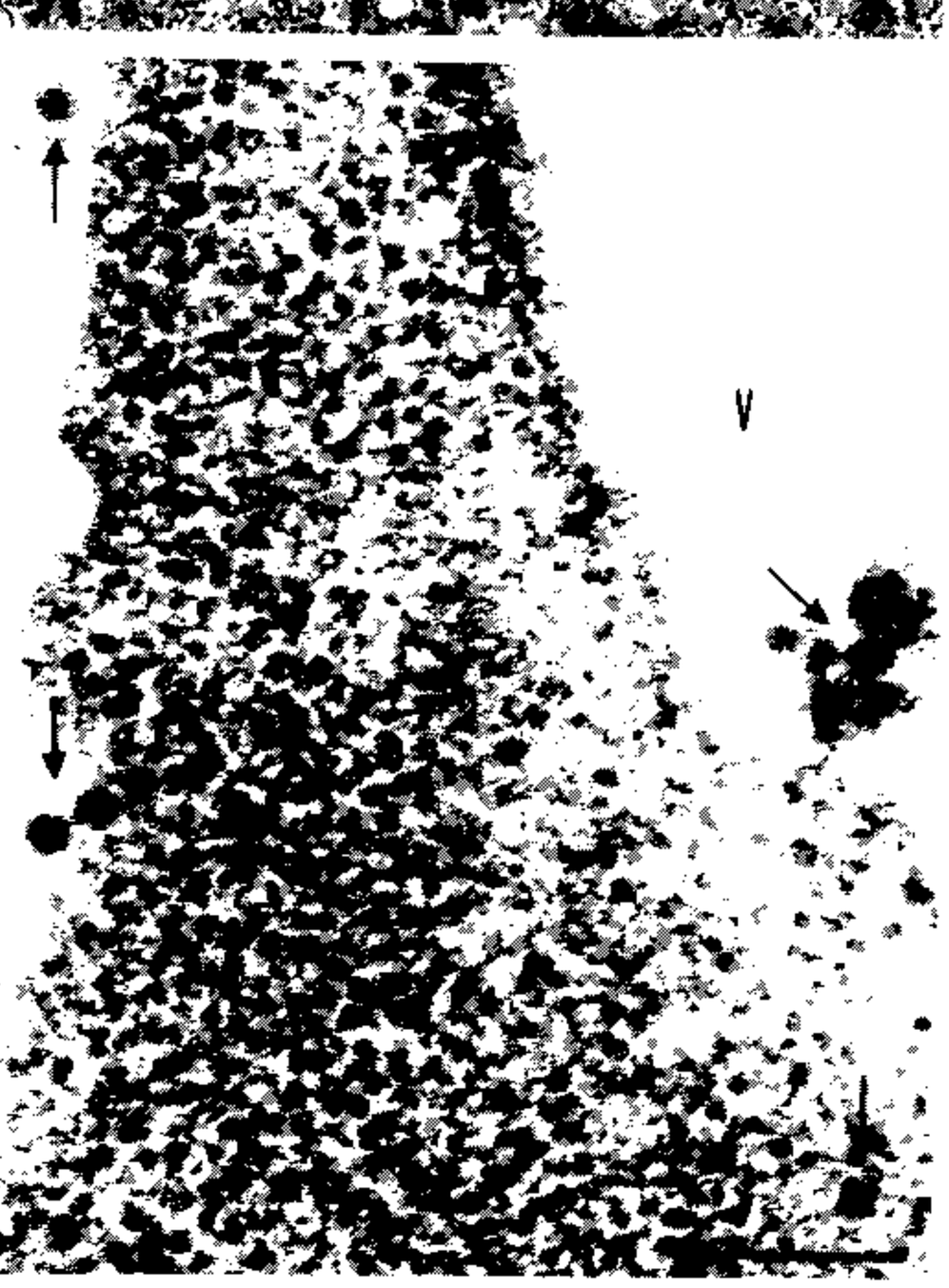


Fig. 5: first group of new virus particles and SMS formed inside the RER, bar = 500 nm. Fig. 6: partially ribosome-coated RER vesicles with newly formed virus particles and SMS, bar = 500 nm. Figs. 7 and 8: virus particles (arrows) inside nuclear membranes at syncytia, bar = 200 nm. Fig. 9: virus particles (arrows) attached to the cell membrane, free inside the cytoplasm and vacuole (V), bar = 200 nm.

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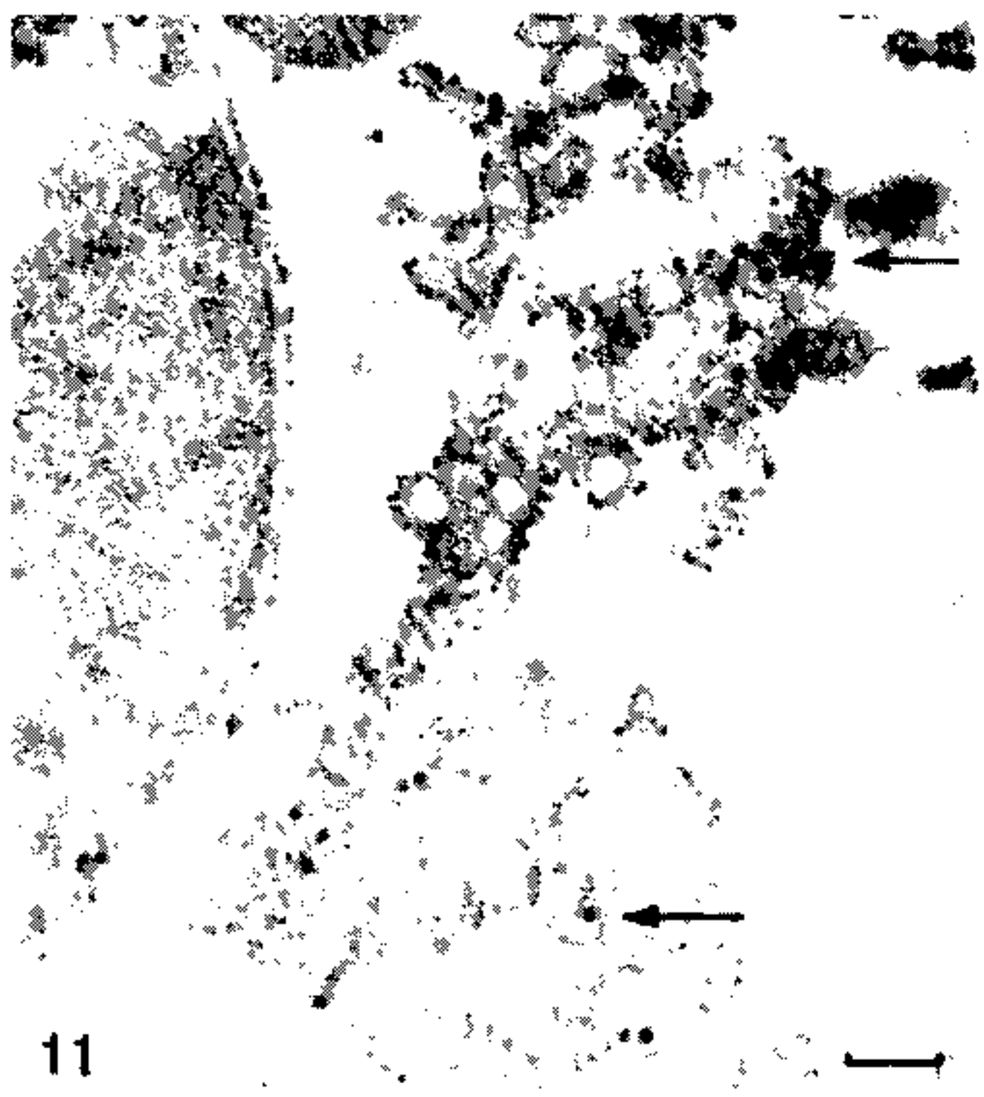

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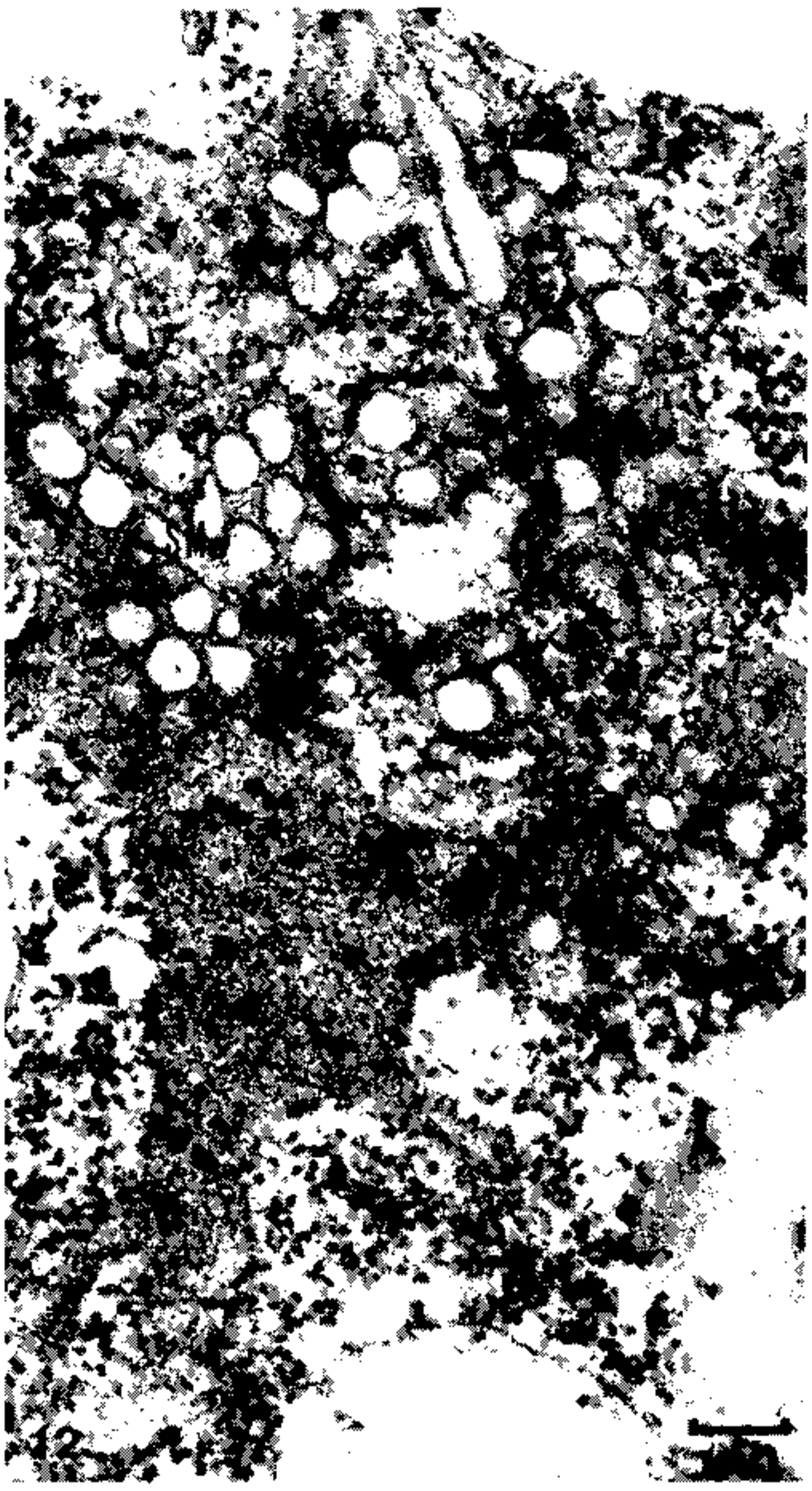

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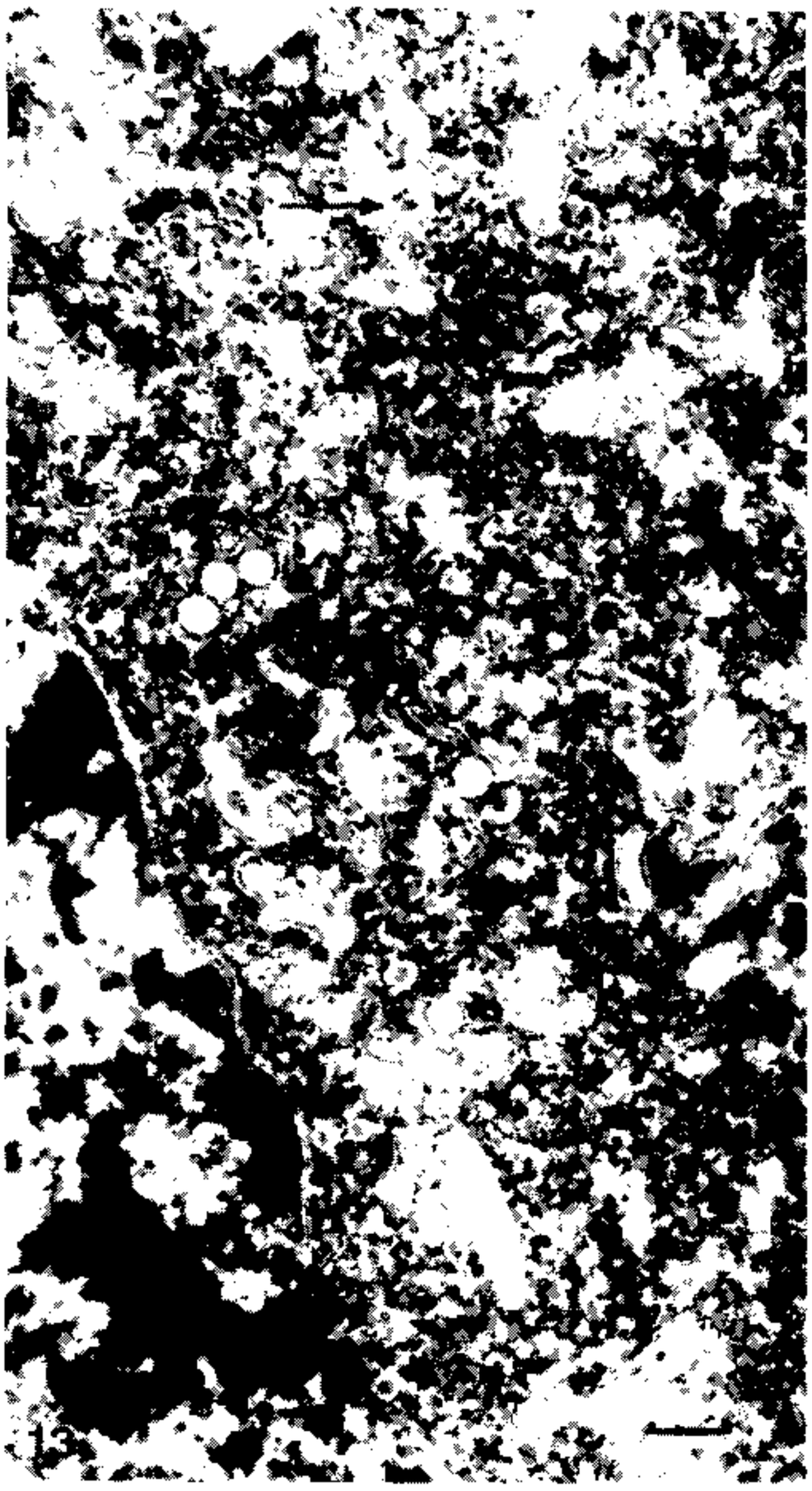

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Fig. 10: two virus particles inside Golgi vesicles; viral RNA contrast enhanced by EDTA treatment. Fig. 11: cell and syncytia lysis; the virus particles (arrows) remain inside cell vesicles. Fig. 12: distended RER with numerous virus particles inside a dense matrix; nucleocapsids contrast enhanced by EDTA treatment; SMS without any contrast modification. Fig. 13: SMS vesicles and tubules without any visible internal structures after alcoholic PTA treatment; no osmic tetroxyde was used; arrow = virus particle. Bars = 200 nm.

vesicles that, like the virus-containing vacuoles and lysosomes, never fuse with the plasma membrane to release virus particles from the cell (Fig. 11).

Dengue-1 virus and SMS characterization -Electron-dense condensations on the cytoplasmic side of the RER membranes suggest the accumulation of viral RNA and proteins to be transfered inside the RER-cisterns. The first detectable virions occur as groups of smooth particles in paracrystaline or linear arrangements in these compartments; single virus particles are seldom observed. By the use of Bernhard's EDTA-technique, small groups of enveloped virus particles with enhanced contrast to its nucleocapsids could be observed inside the RER, together with unalterated SMS. Isolated virus particles inside Golgi vesicles become also evident by this technique (Fig. 10). Sometimes, portions of the distended RER are filled by a dense matrix containing virions (Fig. 12); no structured viral precursors or particles are found inside the SMS.

By the alcoholic PTA treatment for detection of basic proteins, the viral envelop and cell membranes were only gently stained; ribosomes, nuclei and viral RNA are strongly contrasted; the SMS appear always empty (Fig. 13).

Cerium chlorid treatment demonstrate the presence of acid phosphatase inside the cell lisosomes (Fig. 4); no relationship with SMS could be established.

Golgi vesicles may be evidenced by an intense osmium treatment of the cells; they remain electron translucent with very dark and diffuse membranes; also, no relationship with the SMS was found.

DISCUSSION

Virus replication – Dengue-1 virions assemble inside the RER of C6/36 cells. Following the trans-type maturation, some virus particles are transferred to Golgi vesicles (Hase et al., 1989b); rarely they can be detected inside exocytosis vesicles by electron microscopy. In dengue-2 C6/36 infected cells, most virions appear in clatrin-coated transport vesicles, what is more frequently observed in ultrathin sections (data not shown). Electron micrographs provide evidence that free cytoplasmic virus particles can also penetrate the cell membrane directly, as observed here with free virus particles found inside the cytoplasm of dengue-1 infected mosquito cells, similarly to the previously described mechanism for the entry of dengue-2 and Japanese encephalitis viruses into C6/36 mosquito cells (Hase et al., 1989a), although their infectiveness is questionable. All the remaining virus particles produced by the cell remain inside the RER derived vesicles during trans-type maturation and are never released during cell and syncytia lysis into the culture medium.

The cis-type maturation of dengue-2, PR-159 strain, as described by Hase et al. (1989b) is similar to the alphavirus replication (Mezêncio et al., 1989), for which budding from membranes and virus-membrane fusions occur, and was not observed for any of the Brazilian dengue-1 isolates examined.

Smooth membrane structures and cytochemical staining – Little is known about the nature of SMS, which are numerous inside the RER during virion formation at the trans-type maturation. Virus replication is initiated simultaneously with the SMS appearance inside the RER and in the nuclear membrane sometimes; SMS are never observed outside the RER, even with cell lysis. At the end of dengue-1 virus replication, a great number of these structures fill the RER.

The cytochemical reactions applied to mosquito cell cultures infected with dengue-1 viruses, are not very informative on SMS nature. The translucent appearance of its matrix, presenting some internal filaments, is not modified by EDTA treatment; by basic protein staining or osmification of membranes, these filaments are not better emphasized. After cerium chlorid treatment, a dense matrix in the RER, including virus particles, is sometimes detected. There, the virion nucleocapsids are also enhanced by Bernhard's EDTA technique, but no SMS are detected inside these RER cisternae. The application of RNase on ultrathin sections (data not shown) has given, at the moment, no confident new informations. Therefore, the nature of the virus-induced SMS remains unexplained.

Syncytia – Their formation in *A. albopictus* cells infected with flaviviruses is a frequently observed morphologic alteration, but only few

papers reported its occurrence (Ng, 1987; Westaway, 1987). More information about its virus-induced nature are needed, as the syncytia may be formed by different mechanisms of cell fusion and successive nuclear divisions and as they appear to be the prefered sites for virus particles replication.

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