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Ultrastructural Studies on Dengue Virus Infection of Human Lymphoblasts

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Ultrastructural studies of dengue-2 virus-infected lymphoblastoid Raji cells showed that the virus induced an increase in the size of the rough endoplasmic reticula (RER) and that the replication of the virus was confined to the cisternae of these RER. The proliferating RER formed cytoplasmic inclusions that could be seen by light microscopy. This observation could be used as evidence of a cytopathogenic effect of dengue virus on infected Raji cells in routine cultures. Accumulation of virions in the infected cells was minimal in comparison with other cell systems, however. Sporadic clusters of mature virions were often seen on the plasma membrane. These extracellular virions were distributed adjacent to the virus-bearing RER and were presumably released virions. Vertical transmission of the virus was evident in mitotic lymphoblasts. The replication pattern of dengue virus in lymphoblastoid cells suggests that efforts should be made to determine whether blast-transformed lymphocytes, numerous in secondary dengue infections, support dengue virus replication in vivo.

Some lymphoblastoid cell lines have been shown to support the growth of dengue virus (17, 18). In the present study, cellular events during the replication of dengue viruses in a synchronous infection of the Raji lymphoblast cell line were investigated. The findings are viewed in the context of possible interaction between dengue virus and human lymphocytes.

MATERIALS AND METHODS

The Raji cells were infected and embedded in the laboratory of S.B.H. and A.R.D. in Hawaii. The light and electron microscopic studies were carried out in the laboratory of S.S. and N.B. in Bangkok.

Cells. Raji cells (11) were obtained from Werner Henle of Children's Hospital, Philadelphia, Pa. They were propagated in stationary culture bottles (Falcon Plastics) as described previously (17).

Virus. Dengue type 2 virus (dengue-2) strain 16681 was isolated originally by Halstead et al. (8) from the serum of a dengue hemorrhagic fever patient from Bangkok. The virus preparation used in this study was passaged three times in LLC-MK2 cells and two times in Raji cells.

Infection of Raji cells. Raji cells were suspended to a concentration of 3×10^7/ml. To this cell suspension, an equal volume of dengue virus preparation containing 6×10^6 plaque-forming units/ml was added to make an input multiplicity of infection of 0.5. Infected cells were incubated at 37°C for 90 min. During this incubation period, cells were agitated frequently for maximal adsorption. Infected cells were centrifuged at 1,000 rpm for 10 min; then unadsorbed virus was removed, and cells were washed twice to eliminate traces of unadsorbed virus. The infected cells were adjusted to a concentration of 10^6/ml with the growth medium. A replicate set of tubes was made, and cells were incubated at 37°C in an incubator. At various time intervals, supernatant and cells were harvested from one of the replicate tubes for virus growth. A smear of infected cells was made from the replicate tubes and fixed in acetone for immunofluorescent staining. The remaining cells were centrifuged at 1,000 rpm for 10 min, harvested, and suspended in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for electron microscopic studies.

Electron microscopy. Glutaraldehyde-fixed cells were washed in 0.1 M sodium cacodylate buffer (pH 7.2), suspended in 1% osmic acid in Veronal buffer (pH 7.2) for 2 h, dehydrated in graded alcohol, and embedded in epoxy resin. One-micrometer sections were stained with toluidine blue O and pyronin for light microscopic observation. Thin sections were stained with uranyl acetate and lead citrate and examined in Hitachi HS-8 and HU-12A electron microscopes.

Virus assays. Dengue virus was assayed by the plaque technique with LLC-MK2 cells as described previously (8).

Immunofluorescent staining. The technique used for immunofluorescent staining was similar to that described previously (17).

RESULTS

Newly synthesized virus was detected 24 h postinfection in the cells and the supernatant.

Cell-associated virus and virus in the supernatant medium increased exponentially until 72 h postinfection; after that period, there were more viruses present in the medium than associated with cells (Fig. 1). Dengue antigen was detected by immunofluorescence in the cells at 24 h postinfection, when about 15% of the cells showed fluorescence. The percentage of fluorescent cells increased from 15% to about 30 to 40% in the next 48 h and remained at that level during the remaining period of observation. These results were similar to those described earlier (17).

Light microscopic findings. The uninfected lymphoblasts were irregular in shape and contained one to two prominent nucleoli and a moderate amount of cytoplasm. In the infected cells, changes were first observed at 24 h postinfection, when about 10% showed one to two inclusion-like structures in their cytoplasm. The size of these inclusion bodies varied in each cell. There was no nuclear change (Fig. 2). The number of such lymphoblasts increased with time postinfection and was maximal at 72 h postinfection, when approximately 30 to 40% of the cells contained inclusion bodies. The inclusion bodies were later shown to be the sites of viral replication by electron microscopy. At 96 h and subsequent intervals, more necrotic cells were observed, and their number apparently increased with time. By 216 h, about 80% of the cell population was necrotic.

Electron microscopic findings. In the infected and uninfected lymphoblasts, the appearance of the nuclear and nucleolar components was comparable. Structural differences were seen mainly in the cytoplasm. The rough endoplasmic reticulum (RER) of the infected cells was greatly increased in size. This hyperplastic RER was often aggregated into a large body corresponding to the inclusion-like structures observed in the toluidine blue-stained sections. Vesicular structures, mature and immature virus particles, and variants of immature virus particles in different stages of morphogenesis were seen in the dilated cisternae of these RER (Fig. 3). Replication of the virus was limited to the aggregates of RER. In neighboring areas, medium to large vesicles of Golgi complexes were observed. They contained mostly mature virus particles (Fig. 4). Variant particles seen in the cisternae of the RER often showed central dense spots surrounded by varying outer structures. Some of these particles were extraordinarily large and displayed radial spines (Fig. 5 and 6).

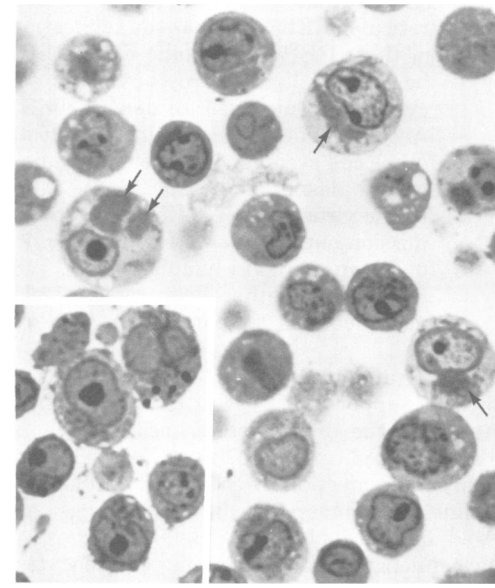

FIG. 2. *Dengue-2-infected Raji cells at 72 h postinfection. One or two intracellular inclusion-like structures are present in the cytoplasm (arrows). Inset: Untreated Raji cells, embedded in epoxy and stained with toluidine blue.* ×*1,600.*

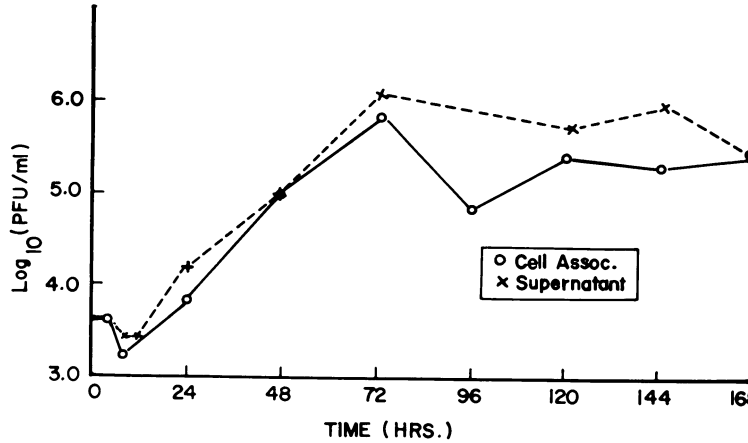

FIG. 1. *Growth curve of dengue-2 16681 Raji-adapted virus in normal Raji cells.*

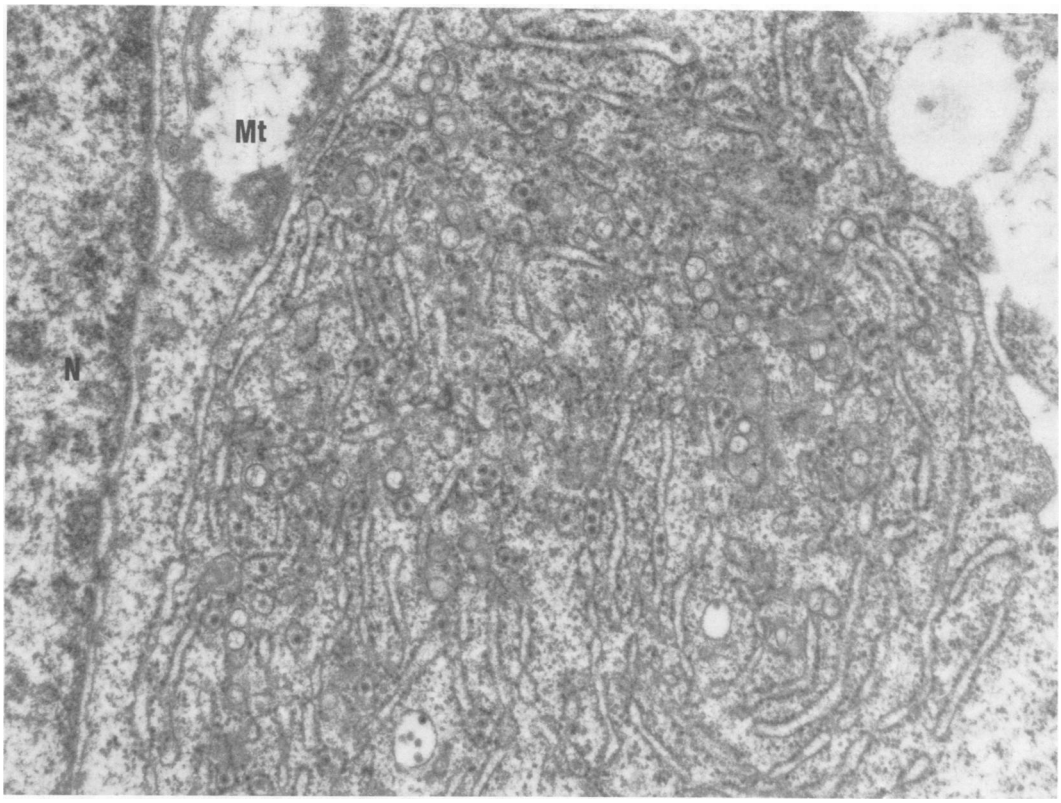

FIG. 3. *Electron micrograph of dengue-2-infected Raji cell demonstrating the aggregated hyperplastic RER containing virions and typical vesicular structures within the distended cisternae. N, Nucleus; Mt, mitochondrion. ×25,000.*

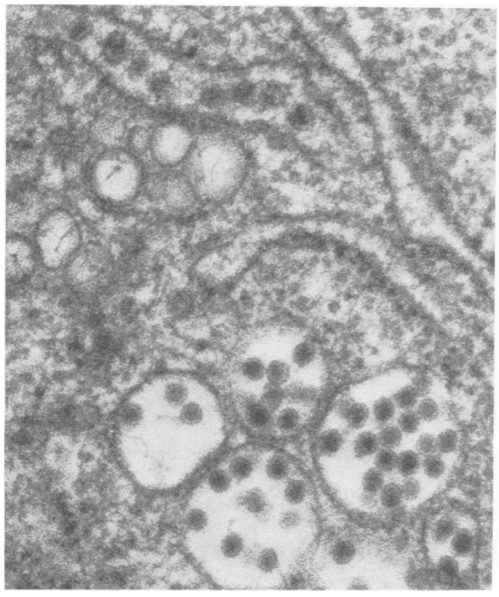

FIG. 4. *Dengue-2-infected Raji cell. Golgi vesicles contain mostly mature virions; the RER cisternae contain mainly immature particles. ×76,000.*

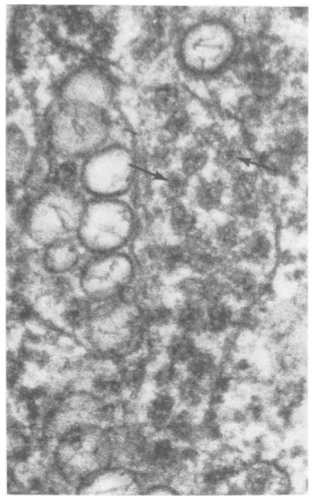

FIG. 5. *Dengue virus particles that may be or are presumed to be in a stage of morphogenesis. Most of the particles consist of a dense core (arrows) and less osmiophilic outer structures. ×76,000.*

Clusters of mature spherical virions were seen on the surface of the cells overlying areas where

RER aggregates with replication of the virus were present in the cytoplasm (Fig. 7). These extracellular virions were not seen on the cell surface where RER aggregates were absent. The immature virus particles were never seen outside the cells, even though they were sometimes located close to the cell membrane (Fig. 8). Replication of the virus was also observed in the cytoplasm of cells in mitosis (Fig. 9). Occasionally, some osmiophilic tubular arrays of the type described previously in the RER cisternae of cells infected with herpes simplex virus, in systemic lupus erythematosus, in neoplasms, and in a number of virus-induced disorders (1) were seen in the RER cisternae of the lymphoblasts, regardless of the presence of dengue virions. In necrotic cells, accumulation of the virus was rarely seen. These cells showed condensation of the nuclear chromatin, disruption of the cell membrane, swelling of the mitochondria, and aggregation of vesicular structures.

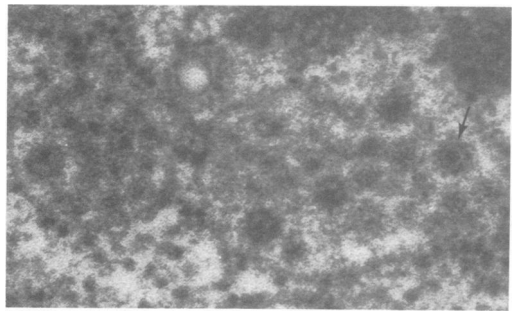

FIG. 6. *Dengue virus particles in a different stage of morphogenesis. Some particles are extraordinarily large and have radial spines (arrow).* ×*76,000.*

DISCUSSION

From the present study, the dengue virus appears to induce an increase in the size of the RER system of the lymphoblasts as a prerequisite for the replication of the virions. This is more evident when the cellular events are compared with those observed in lymphoblasts naturally infected or inoculated with Epstein-Barr virus or other herpesviruses. The RER of the herpesvirus-infected lymphoblasts are intact;

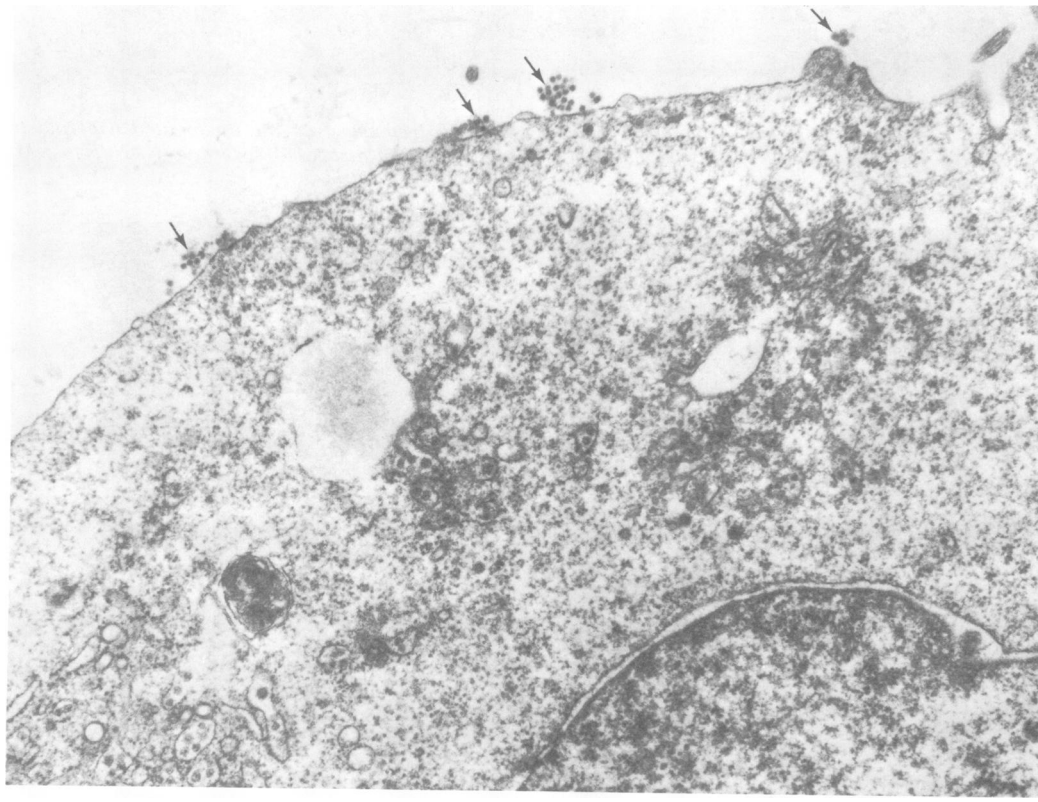

FIG. 7. *Low-power electron micrograph of an infected Raji cell showing four clusters of dengue virions on the cell surface (arrows) adjacent to the large and small aggregates of RER, where virus replication is seen. There is no extracellular virion present on other areas of the cell surface.* ×*23,500.*

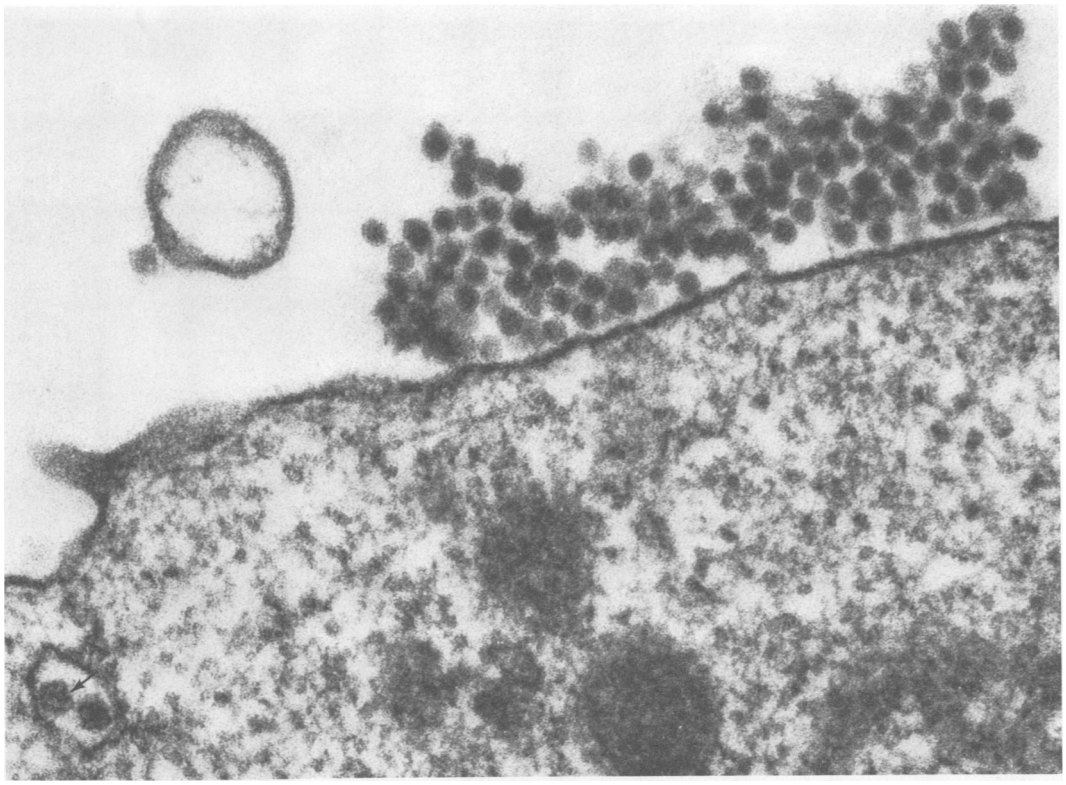

FIG. 8. *High-magnification micrograph of dengue virions on the surface of the infected Raji cell. Others in the cisterna are immature particles (arrow). ×95,000.*

the viruses are assembled in the nucleus and mature by budding through the nuclear or cell membrane (5, 6). The morphology of the virions, the site of viral assembly, and the mode of replication in the present study were all indicative that the virions that replicated in the Raji cells were solely dengue virus. No virion suggesting Epstein-Barr virus or herpesviruses was seen. The latent Epstein-Barr virus genomes in the Raji cells apparently do not interfere with the replication of dengue virus.

The morphology of mature virions produced by the Raji cells was identical to that of the mature dengue virions produced in cultured mammalian cells and suckling mouse brains (9, 10, 15–18). Similarly, granular structures seen on the viral envelope produced in mosquitoes (14) were not seen in Raji cell material. Dengue virions produced in the Raji cells were less osmiophilic than the virions produced in mouse neurones in our previous studies (15, 16). The variant immature virus particles appeared to show more variations than those forms produced in other types of cells. However, the central dense spots (similar to those seen in the filaments [15 to 25 nm in diameter]) that had been proposed to be the ribonucleic acid element of the dengue virus core (15, 16) were again consistently observed in the variant virus particles (Fig. 5 and 6). This consistency in appearances strengthens our belief that the mode of virus morphogenesis is basically similar in different hosts. The varying particulate structures may result from the smaller amount of lipoprotein content in the virus produced in the Raji cells. The lesser degree of osmiophilia helps in the unmasking of structures that are usually masked by the osmiophilic components. Smith et al. (13) noted a lower buoyant density of mouse brain-derived virions in comparison with the virions derived from kidney cell cultures. They concluded that there probably was more lipoprotein in the brain tissue-derived virions. Failure of high mouse brain-passaged dengue-2 to produce viremia in monkeys and to replicate in Raji cells has been observed (7, 17), and it is not clear at present whether the reported noninfectivity was due to the virus or to inhibitory substances from the mouse brains.

The clusters of virus on the cell surface were presumably virions released during the productive phase. The Golgi vesicles could represent

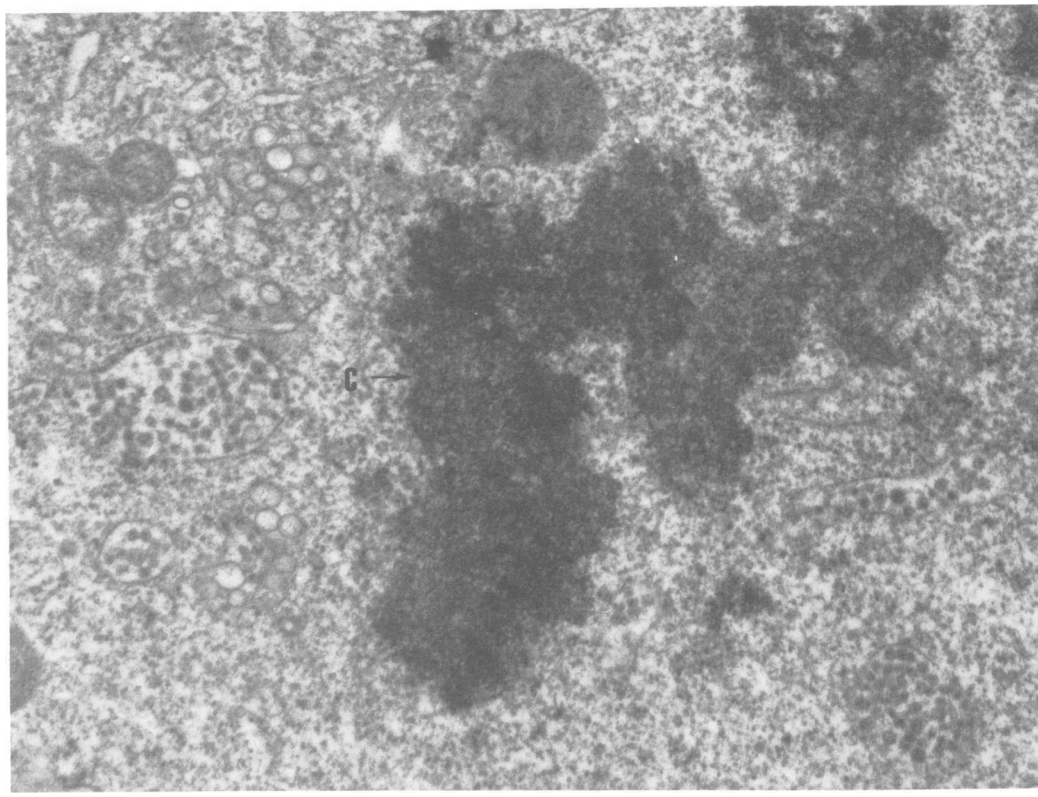

FIG. 9. *Infected Raji cell in mitosis. Distended RER cisternae with virus particles and vesicular structures are seen in the cytoplasm on both sides of the chromosomal elements (C). ×30,500.*

pools for the mature virions. The evidence further suggested that the virions were gradually released from the cells, for there was no accumulation in the necrotic cells. This could also account for the fact that virus titers were higher in the culture medium than in the cell-associated fractions. It is possible that the release could be fairly rapid, but the actual process could not be well documented. The observation of replication of virus in mitotic cells indicates the possibility of vertical transmission as a mechanism in addition to cell-to-cell spreading.

The replication of dengue virus in Raji cells differed from that in other permissive cells (9, 10, 14–16) in two minor features: (i) Crystalline-arranged viral aggregates were rarely present in the Raji cells, and (ii) quite a large number of the virus particles located in the RER were immature, whereas those located in the Golgi vesicles and extracellularly were always mature virions. These observations are consistent with the findings of others working with lymphoblasts, who found virus-like particles within the perinuclear membranous complexes of RER in the early stages of dengue virion formation (7), but identification of complete virions was very difficult. This contrasted with the intense cytoplasmic staining in immunofluorescent observations (18). Why immature virions are so numerous in the infected lymphoblasts is not known.

The variant immature particles were only seen intracellularly and were larger than the mature virions, which were found intra- or extracellularly. The size difference between intra- and extracellular virions has not been reported in previous studies. In our experience with dengue virus replicating in neurones of the suckling mouse and mosquito, some immature particles of unusually large size have been observed, but in much smaller numbers than in Raji cells (Fig. 2 of reference 14; Fig. 11 of reference 16). A review of the electron microscopic studies of other investigators reveals variation in the size of intracellular virions in dengue-infected LLC-MK2 cells (Fig. 5 of reference 3). Where the relative location of intra- and extracellular dengue virions are shown, the virions are well assembled and of consistent morphology (4, 9, 10). Examination of the electron micrograph of Matsumura et al. (9, Fig. 3) reveals a few virus particles with faint radial spines in a RER cisterna. It may be pertinent to recall that the viral

membrane (envelope) protein composition of flaviviruses changes significantly during morphogenesis. The intracellular form of the virus contains two membrane glycoproteins, one of which disappears from the extracellularly released virus and is replaced by a nonglycosylated membrane polypeptide. This suggests cleavage of the glycoprotein in the late stage of morphogenesis, after the intracellular form of the virus is assembled, to form the nonglycosylated membrane polypeptide (12). Morphological differences between immature and mature virions in Raji cells suggest that maturation occurs within the cisternae of the endoplasmic reticulum, probably before the virion is transported to the Golgi vesicles. If the intracellular (immature) and extracellular (mature) virions truly differ in membrane protein content, elimination of excess membrane during morphogenesis (12) might remove the radial spines or any excess membrane from the immature virus.

The intracytoplasmic RER aggregates were well defined in the toluidine blue-stained sections. The presence of inclusions in light microscopic sections of the dengue virus-infected Raji cells has not been noted in previous studies. The consistent appearance of these inclusions, indicative of cells infected with dengue virus, could be useful in routine cell culture work.

The productivity of the infection is proportionate to the amount of proliferating RER. The latter phenomenon, at least in our previous experimental studies with mouse neurones and mosquito systems, is virus induced. Evidence has been developed that suggests that lymphoblastoid cell lines of B cell origin and monocytes (macrophages) support dengue virus replication (7, 18), but mitogen-transformed B lymphocytes may also support dengue replication (18). Despite the previous observations of lymphocytosis (both B lymphocytes and transformed lymphocytes) as well as the observation of dengue antigen on the surface of lymphocytes from patients with dengue hemorrhagic fever (2), the in vivo site or sites of dengue virus replication are not known with certainty. Efforts should be made to determine how these cell types support dengue replication in human dengue infection.

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