Morphogenesis of Yellow Fever Virus 17D in Infected Cell Cultures

By R. ISHAK, 1, 3 D. G. TOVEY 2 AND C. R. HOWARD 1 *

1 Department of Medical Microbiology and 2 Electron Microscopy Unit, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, U.K. and 3 Universidade Federal do Pará, Caixa Postal 3005, 66000-Bélem-Pará, Brazil

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SUMMARY

The morphogenesis of yellow fever virus replication was examined in infected Vero cell cultures. Penetration and uncoating occurred by endocytosis with the formation of coated vesicles, similar to that demonstrated for other enveloped and unenveloped viruses. Inclusion bodies associated with newly formed nucleocapsids were evident in the perinuclear region during the growth cycle. No evidence of RNA synthesis in the vicinity of the inclusion bodies was obtained by autoradiography, suggesting that genome replication and assembly of viral nucleocapsids occur at separate cytoplasmic sites. An excessive proliferation of membrane-bound organelles involving both vacuoles and endoplasmic reticula was the most striking feature of virus-infected cells late in infection. No morphological changes in the appearance of nuclei or mitochondria were detected. Virus release appeared to occur by movement of nascent virions through the proliferated endoplasmic reticula followed by exocytic fusion of virus-containing vesicles with the plasmalemma. A possible mechanism whereby the internal nucleocapsid acquires an outer envelope is discussed.

INTRODUCTION

Yellow fever in man varies from an inapparent infection to a fulminating disease which is invariably fatal (Smetana, 1962). Despite the effectiveness of mosquito eradication in reducing the incidence of infection and the availability of an effective vaccine, epidemics continue to occur, particularly in Africa (World Health Organization, 1971; Carey et al., 1972), and there is some evidence of foci of infection maintaining a reservoir between epidemics (Monath et al., 1974).

The virus of yellow fever (YFV) is the type species of the genus Flavivirus and of the family Flaviviridae which includes over 30 members pathogenic for man and vertebrates (Westaway et al., 1985). Yellow fever is an infection principally of primates and is transmitted from man to man by Aedes mosquitoes. However, little is known regarding the pathology of this infection in either man or laboratory animals. Clinically, infection involves the spleen, kidneys and heart as well as the liver. Infected hepatocytes show a number of characteristic changes, the most prominent being the formation of Councilman bodies resulting from acidophilic degeneration. The appearance of these inclusion bodies correlates well with virus replication and precedes biochemical and histological signs of liver cell damage.

Yellow fever virus is enveloped, of average diameter 38 nm, and is indistinguishable from other flaviviruses on morphological criteria alone (Bergold & Weibel, 1962; Deubel et al., 1981). Only limited studies have been performed as to the ultrastructural changes accompanying YFV replication. This has been associated with a proliferation of cytoplasmic membranes with the accumulation of nascent virus particles within the cisternae of the endoplasmic reticulum (ER) or inside large vacuoles. The nature of the virus-specific inclusion bodies, the mode of virus entry and the mechanism of virus maturation are unclear, however. In the present study, the morphogenesis of a vaccine strain (17D) of YFV has been examined in acutely infected cell cultures.

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METHODS

Cell cultures. The Vero and BHK-21 cell lines were used as substrates for virus growth. Cultures of Vero, BHK-21 and mouse L929 cells persistently infected with YFV, substrain 17D-204, were established in this laboratory by C.P. Itobi in 1983 and maintained by sequential weekly subculturing.

Virus strains and growth. Yellow fever virus substrate 17D was used to infect either BHK-21 or Vero cell cultures at an m.o.i. of 0.1 p.f.u./cell. The virus stock originated from a vaccine preparation (Vacina Anti-Amarilica, lot 182A) produced by Fundação Instituto Oswaldo Cruz, Biomanguinhos, Rio de Janeiro, Brazil (kindly provided by Dr. O. Souza Lopes). The virus was initially passaged in Vero cells and then cloned three times. Virus from isolated plaques in Vero cell monolayers was amplified in BHK-21 cells before recloning in Vero cells.

Electron microscopy. Thin section electron microscopy was performed essentially as described by Ellis et al. (1984). Electron microscopy autoradiography was carried out using the method of Tovey & Kendall (1979) with some modifications. Briefly, sections were cut onto a collodion-coated glass slide, impregnated with 'Amplify' (Amersham) for 15 min, covered with a thin layer of carbon and further coated with film emulsion Ilford L4 diluted 1:3 in distilled water. The slides were kept in a light-proof box containing silica gel at 4 °C for an appropriate period of time and then developed with developer D19 (Kodak) for 3 min at 20 °C. Following a brief wash in distilled water, the emulsion films or slides were fixed in 25% sodium thioupsulate for 1 min and floated onto 200-mesh copper grids. Saturated uranyl acetate in 100% methanol was used for staining. Grids were immersed for 30 min in the stain, briefly washed twice in 95% methanol, and then immersed in lead citrate pH 12, for 10 min. The grids were examined in a JEOL model 100CX transmission electron microscope.

Timetable of cell infection and harvest. Cells were infected sequentially in two sets of experiments. In the first the infected cells were fixed and processed within 30 min post-infection; in the second, cells were harvested from the first to the fifth day post-infection. Persistently infected cells were harvested on the sixth day after subculture.

RESULTS

Adsorption, penetration and uncoating

Thin sections of infected Vero cell cultures were examined within the first 30 min of infection in order to elucidate events. An initial thickening of the plasmalemma at the site of virus adsorption was followed by the appearance of pedicular attachments between the virus and the membrane (Fig. 1).

Contact between the virus and the cell membrane appeared to trigger the uptake of the virus exclusively by a process of endocytosis; other possible routes and modes of entry were not detected. The formation of a membrane invagination with the appearance of a coated pit was seen to precede the encirclement of the virus particle (Fig. 1 d). Virus particles were carried further into the cytoplasm within coated vesicles after the thickening of the plasma membrane in the area of virus-containing vesicles (Fig. 1 c).

The release of the nucleocapsid, either complete or devoid of RNA, from the coated vesicle into the cytosol was not clearly resolved. Cytological changes may accompany genome penetration as indicated by the following observations. Firstly, extensive fusion of coated vesicles together with larger cytoplasmic vacuoles was seen accompanied by the aggregation of internal complete virus particles. Secondly, virions in the process of losing the viral envelope and the nucleocapsid appeared to undergo disaggregation within otherwise complete vesicles (data not shown). This may result in the loss of structural integrity between subunits and release of the nucleocapsid although this was not clearly seen in the present study.

Assembly and release of nascent virus

Vero cell monolayers infected with YFV were examined at regular intervals until the fifth day post-infection. After 24 h mature virus particles were seen, both within cytoplasmic vacuoles and as extracellular virus aggregates. Infectious virus was released continuously until cytolysis.

Vacuolization was the most prominent feature on the first day post-infection (Fig. 2a). The second and the third days post-infection were characterized by the appearance of virus particles, mostly inside cytoplasmic vacuoles and the ER (Fig. 2b). Vacuoles of various sizes were distributed throughout the cytoplasm, particularly around the nucleus. These virus-containing vacuoles tended to fuse with larger vacuoles prior to transport of the virions into the extracellular environment. During these phases of virus growth, the Golgi complex became sparse and difficult to detect compared to uninfected control cells. In contrast, the mitochondria and the
nucleus remained unchanged. Single virus particles could be seen in the process of transport and release either through a vacuole or the ER. Extracellular, isolated particles appeared to form large aggregates (Fig 3c). Frequently, virions were also seen within dilated regions of the perinuclear space (Fig. 3a), but virus particles were rarely organized into symmetrical crystalline arrays.

Membraneous structures formed into an amorphous mesh of converging ER membranes (Fig. 4) within which nucleocapsid-like structures and virions frequently protruded into cytoplasmic cisternae. The area for nucleocapsid assembly could not be clearly established, however. Although virus particles were present in most areas of the infected cell, a concentration existed in and around the membraneous amorphous material which contained nucleocapsid-like structures (Fig. 4). In addition, these inclusion-like areas were usually located in close proximity to the cell nucleus, an area which rapidly degenerated as the infection progressed. Electron microscopy autoradiography showed the deposition of [\(^{35}\)S]methionine and [\(^{3}\)H]glucosamine, but not [\(^{3}\)H]uridine, in and around these inclusion-like structures (Fig. 5). Nucleocapsids were
Fig. 2. Ultrastructure of uninfected (a) and YFV-infected Vero cells (b). The latter shows the proliferation of membrane-bearing organelles, the presence of virus particles inside vacuoles and a distended ER. Bar marker represents 50 nm.
Fig. 3. Release of YFV from infected Vero cells. Virions were present within the perinuclear space (a) and as aggregates both within the cytoplasm (b) and after release (c). Electron-dense structures on the cytosolic side of the membrane may represent nucleocapsids. Bar marker represents 50 nm.
Fig. 4. Assembly of YFV in Vero cells. Virus-containing ER with virus and nucleocapsids abutting onto the membrane are shown in (a), and a virion within the ER together with a nucleocapsid emerging from the membrane in (b). Nucleocapsids concentrated onto the cytosolic side of the ER membrane can be seen (c). Bar marker represents 50 nm.
Fig. 5. Autoradiography of YFV-infected Vero cells after pulse-labelling with $[^{35}\text{S}]$methionine (a), $[^{3}\text{H}]$glucosamine (b) and $[^{3}\text{H}]$uridine (c). Bar marker represents 50 nm.
not found associated with the plasmalemma and complete virions were not in direct contact with the cytosol. It appeared that both the richly developed vacuolated area and the canalicules formed by the ER contributed to the centrifugal movement of the virus from the centre of the cell to the external medium. The cells at the fourth and fifth day post-infection showed a similar profile of cytological changes, although by this stage of infection cells had progressively degenerated or lysed.

Few virus particles were seen in the process of acquiring an outer envelope. It appeared that during this process the nucleocapsid became flattened as protrusion began through the membrane, with the virus outer envelope encircling the nucleocapsid. Appendages and links between the envelope and the inner side of the organelle membrane were frequently present (Fig. 6).
**DISCUSSION**

Internalization of YFV follows a mechanism similar to that reported for other enveloped and unenveloped viruses (Helenius et al., 1980; Yoshimura & Ohnishi 1984; Svensson, 1985; Zeichhardt et al., 1985), with virus particles entering predominantly by endocytosis (Fig. 1 and 2). Enveloped viruses are thought to release their nucleocapsid into the cytosol by fusion of the envelope with the inner lysosomal membrane (Marsh, 1984), although this mechanism of release remains largely unknown. It has recently been suggested that West Nile flavivirus accumulates within endosomes prior to the occurrence of the fusion (Gollins & Porterfield, 1985), and a similar mechanism appears to be involved for YFV. However, the finding of vesicles containing YFV in the process of disintegration (data not shown) suggests the release of the infectious RNA occurs within the endosomes.

Extensive proliferation of membraneous organelles appears to be a unique feature of flavivirus-infected cells (Murphy, 1980), with large numbers of vesicles already apparent by the end of the eclipse period (Ota, 1965; Matsumura et al., 1971). This excessive production of membranes may be either induced or controlled by the virus, or both. The proliferation of membrane-bound organelles, in particular the ER and vacuoles, would favour both virus protein production and glycosylation, together with an enhancement of virus release involving the exocytic pathway. Yellow fever viral glycoproteins are thus likely to be synthesized by membrane-bound ribosomes attached to the ER. The association of flavivirus proteins with membranes (for review, see Westaway, 1980) could be explained by the extensive proliferation of membranes in the infected cell. It should be noted that not all envelope proteins are glycosylated, e.g. Schlesinger et al. (1983) found an unglycosylated YFV envelope protein which conceivably can be synthesized on non-membrane-bound ribosomes.

Flavivirus RNA synthesis and RNA translation apparently occur at different sites within the infected cell (Westaway & Ng, 1980; Ng et al., 1983). Yellow fever virus RNA synthesis appears not to be restricted to any one area of the cell; in contrast protein synthesis appears to be restricted to the perinuclear region. The site for nucleocapsid assembly could not be resolved fully in the present study, although it is likely that the major nucleocapsid protein aggregates around newly synthesized RNA. The inclusion bodies appeared to be closely associated with nucleocapsids (Fig. 4). This is an area rich in membranes and resembles the ultrastructural changes observed previously for both YFV and other flaviviruses (Baruch, 1963; Southam et al., 1964; Murphy et al., 1968). Such inclusions consist of protein material only, and autoradiography of these regions showed the absence of nascent RNA chains (Fig. 5).

Release of the newly formed YFV is achieved by the peripheral movement of virions through the extensively proliferated ER followed by the exocytic fusion of small and large vacuoles containing virions with the plasmalemma (data not shown). This pre-existing excretion pathway developed by the cell is used in much the same way as the virus utilizes the endocytic pathway for entry.

The Golgi apparatus is generally involved in the cellular exocytic pathway, although the direct involvement appeared not to be the case with YFV. The lack of contact with the Golgi apparatus is supported by evidence from studies on Kunjin virus-infected cell membranes (Boulton & Westaway, 1976; Westaway & Ng, 1980; Ng et al., 1983). Sucrose gradient centrifugation showed that Golgi membranes sedimented as a band distinct from those in which glycosylation of viral proteins occurred. The normal cell pathway of formation of glycoproteins containing complex oligosaccharides is from the ER to the Golgi apparatus. However, it has been shown that flavivirus glycoproteins also contain complex oligosaccharide side-chains, suggesting that the production and initial glycosylation of the envelope glycoprotein in the ER is followed by subsequent transport to the Golgi apparatus for further processing, and then insertion into the membrane of Golgi apparatus-derived vesicles. These vesicles would acquire the completed glycoprotein either by fusing with vacuoles or by fusing with the ER resulting in the encirclement of the nucleocapsid with the envelope glycoprotein. The extensive vacuolization within the infected cells could thus be explained by the intracellular transport between two organelles. However, a satisfactory explanation is lacking for the occasional presence of vesicles inside the ER. For example, Japanese encephalitis virus may be observed...
within the Golgi apparatus (Leary & Blair, 1980). It is possible that enclosed vesicles are an artefact due to the plane of sectioning (vesicles were clearly differentiated in the present study, see Fig. 2a); the presence of particles in the Golgi compartment may thus be coincidental as flaviviruses may also be found in the perinuclear space (Fig. 3a).

The origin of the flavivirus envelope and by what mechanism the nucleocapsid acquires this coat is still unclear. Ota (1965) has suggested that Japanese encephalitis virus develops by budding through vacuolar membranes. However, this early finding was later interpreted as representing the discharge product resulting from the fusion of a small transport vacuole with a large one (Westaway, 1980).

In the present work YFV was seen to acquire its envelope through a process of budding (Fig. 6) although it was more common to see appendages and links between the envelope and the inner side of vacuoles and the ER. This process was seen in both Vero and BHK-21 acutely and persistently infected cells. It is not understood why this process is not more commonly seen with YFV and other flaviviruses. The process may occur rapidly owing to the close association of flavivirus protein with intracellular membranes (Shapiro et al., 1972; Stohlman et al., 1975; Boulton & Westaway, 1976). The interaction between nucleocapsids and the single protein component of the envelope may thus be enhanced.

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