Ultrastructural and Biochemical Study of Frog Virus 3 Uptake by BHK-21 Cells

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SUMMARY

Ultrastructural studies of the uptake of enveloped and naked frog virus 3 (FV 3) particles by BHK-21 cells have shown that enveloped viruses are internalized by adsorptive endocytosis via coated pits. The enveloped particles then appear to move through endosomes and finally lysosomes. Naked viruses may also follow the same pathway but only rarely. Their more frequent mode of entry is by fusion between the virus shell and the cellular membranes, thus allowing the virus to shed its nucleoprotein content directly into the cytoplasm. This difference in the mechanism of penetration has been confirmed by the use of lysosomotropic agents: the inhibition of viral growth being far more drastic for enveloped FV 3 than for naked virus implies that a lysosomal step is required for the multiplication of enveloped viral particles.

INTRODUCTION

Frog virus 3 (FV 3), a Ranavirus which belongs to the Iridoviridae family (Fenner & Gibbs, 1983), is a large lipid-containing icosahedral DNA virus (for review, see Goorha & Granoff, 1979). Its multiplication, which takes place in the cytoplasm at temperatures between 26 °C and 29 °C, is relatively slow, since in BHK-21 cells mature progeny viruses only appear from 5 h post-infection onwards (Bingen-Brendel et al., 1971). At later times, FV 3 is released either by budding at the plasma membrane, in which case the particles are enveloped, or by cell lysis in which case they are referred to as naked. The possibility of separating these two populations by physical means allowed us to show previously that enveloped particles are more infectious than the naked ones (Braunwald et al., 1979). The presence of a viral envelope may be an important factor in the early interactions of the virus particles with the plasma membrane. In order to analyse these first critical steps in detail, BHK-21 cells were infected with either enveloped or naked FV 3 and the ultrastructural features of viral uptake were investigated.

A single pathway of virus uptake, adsorptive endocytosis, has been shown to exist in the case of enveloped particles whereas naked particles penetrate mainly by fusion with the cellular membranes. The action of lysosomotropic agents on viral multiplication has also been studied and the results corroborate these different mechanisms of penetration.

METHODS

Cells. BHK-21 cell monolayers were grown at 37 °C in Eagle's medium (BHK-21, Eurobio, Paris, France) supplemented with 10% calf serum. Depending on the experiment, 25 or 175 cm² plastic flasks (Falcon) were seeded with 5 × 10⁶ or 4 × 10⁷ cells respectively, and tissue culture plates (Costar) with 1.5 × 10⁶ cells/well.

Production and purification of naked and enveloped virus particles. Cells grown for 24 h on 175 cm² flasks were infected with FV 3 at an input multiplicity of 0.1 p.f.u./cell. After a 1 h adsorption period at room temperature, the cells were incubated at 29 °C in Eagle's MEM (Eurobio) supplemented with 5% calf serum; the infection was allowed to proceed for about 60 to 72 h until complete necrosis of the cells.

Crude viral extracts, obtained from broken infected cells after a low-speed centrifugation (2500 g for 20 min), were layered onto 43% (w/w) sucrose cushions in 10⁻² M-Tris pH 8.9 and centrifuged at 4 °C for 4 h at 82500 g. The
Table 1. Protocol of chloroquine addition to FV 3-infected BHK-21 cells

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Temperature (°C)</th>
<th>Chloroquine</th>
<th>Titration</th>
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<tr>
<td>-1</td>
<td>4 °C, 29 °C</td>
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<tr>
<td>0</td>
<td>4 °C, 29 °C</td>
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<td>18 h</td>
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pellets were resuspended in the same buffer, then centrifuged on 20 to 65% (w/w) sucrose gradients in 10^{-2} M-Tris pH 8.9 (30000 g, 20 min). Virus bands were collected and subjected to equilibrium centrifugation on 35 to 65% (w/w) sucrose gradients in 10^{-2} M-Tris pH 8.9 (82500 g for 15 h), which separated enveloped viral particles (upper band) from naked viruses (lower band) (Braunwald et al., 1979). Some suspensions of enveloped viruses were treated prior to the experiments with a solution of DNase in phosphate-buffered saline (PBS) at a final concentration of 100 µg/ml for 1 h at 37 °C. As a control, suspensions of naked virus particles were treated in the same way and both samples were titrated.

All preparations were checked before use by electron microscopy.

Virus multiplication: action of lysosomotropic agents. Cell monolayers grown in 25 cm² plastic flasks were infected in a cold room with 0.5 ml of virus suspension at about 5 p.f.u./cell. After 1 h adsorption at 4 °C, they were washed three times with cold PBS, and then incubated at 29 °C with 5 ml MEM containing 5% calf serum. The end of the adsorption step is referred to as time 0 of infection.

Aqueous solutions of 10 mM-chloroquine [7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline, diphosphate salt; Sigma] at a final concentration of 0-1 mM were added to the virus suspension, the PBS and the culture medium either from the beginning of infection or at 1, 3, 5 or 7 h post-infection (Table 1).

Virus titres were determined at 0 h and 18 h post-infection by plaque assay at 29 °C on monolayers of BHK-21 cells grown on 3.5 cm Petri dishes. Controls of virus multiplication were performed in the absence of chloroquine.

Electron microscopy. Morphological studies of the early steps of infection which required high multiplicity of input virus were performed on cell monolayers grown on tissue culture plates infected in a cold room with about 500 p.f.u./cell, under a volume of 50 µl. After 1 h adsorption at +4 °C, the inoculum was removed and the cell monolayers were washed three times with cold PBS. The tissue culture plates were then warmed by floating on a water-bath at 29 °C and 200 µl of 37 °C prewarmed MEM supplemented with 5% calf serum were added; the cultures were then incubated in a room at 29 °C. At various times, before ethanol dehydration and embedding, they were either submitted to a 2-5% glutaraldehyde fixation for 30 min at +4 °C, then to a 2% osmium tetroxide post-fixation for 1 h at +4 °C (batch 1), or to a simultaneous fixation with a glutaraldehyde–picric acid–osmium tetroxide mixture and a uranyl acetate post-fixation (batch 2); this latter procedure, described by Takahashi (1980), enables a better visualization of membranes. They were then processed in situ for electron microscopy (Kawamoto et al., 1980).

In parallel with the titration experiments in the presence of chloroquine, virus multiplication was checked by electron microscopy: cells were grown in 25 cm² plastic flasks and infected at a multiplicity of 5 p.f.u./cell. Prior to processing for electron microscopy, the cells were scraped off and centrifuged.

For the study of fusion at low pH, cells grown in 25 cm² plastic flasks were infected at a multiplicity of about 500 p.f.u./cell under a volume of 0.5 ml. After 1 h adsorption at 4 °C, removal of the inoculum and washes as described above, the cultures were incubated at about 29 °C in 5 ml of 37 °C prewarmed BHK-21 medium at pH 5.5; after 30 or 60 s, they were rinsed with ice-cold BHK-21 medium pH 7.2 and immediately fixed according to Takahashi (1980). The medium at pH 5.5 was obtained by adding a solution of 7% NaHCO₃ to BHK-21 medium pH 4.6 devoid of NaHCO₃.

Thin sections were cut with a LKB Ultrotome III, stained either with uranyl acetate and lead citrate (batch 1) or with lead citrate alone (batch 2) and examined with a Philips EM 300 electron microscope.
Fig. 1. Cells infected with FV 3 and fixed after 1 h adsorption at 4 °C. Viral particles can be seen outside the cell, adsorbed or not adsorbed to the plasma membrane. No viral structures are present inside the cytoplasm. Bar marker represents 1 μm. Inset: viral particle adsorbed to the cell membrane. Bar marker represents 100 nm.
Fig. 2 to 8. Endocytosis of enveloped and naked FV 3. Fig. 2, 3, 4 and 8 show fixation and staining by the method of Takahashi (1980). For other figs, see Methods. All bar markers represent 100 nm.

Fig. 2 to 5. Uptake of enveloped FV 3 following 1 h at 4 °C and further incubation for the indicated times at 29 °C.

Fig. 2. Enveloped viral particle close to a coated pit (45 min).

Fig. 3. Enveloped virion (viral envelope: arrow) near the plasma membrane in a coated vesicle, perhaps still connected with the extracellular space (10 min).

Fig. 4. Deeper in the cytoplasm, a virion inside a vesicle (viral envelope: arrow). Many intracellular membranes are present in its vicinity (1.5 h).

Fig. 5. Two particles inside a secondary lysosome (arrows). Owing to their degree of degradation, it is no longer possible to state whether they are enveloped or naked virions. Only the nucleoid is recognizable (3 h).
Virus particle counts. The frequency of the various types of interactions between naked FV 3 and the cells was determined by counting 300 virus particles interacting with the cell membranes in about 2500 cell sections. The standard deviation was less than 10% which appeared sufficient for valid interpretation of our results. As far as enveloped FV 3 was concerned, the observations of 150 virus particles interacting with the cell membranes were sufficient since they were relevant to only one type of interaction. Particle:infectivity ratios varied from preparation to preparation (Braunwald et al., 1979).

RESULTS

Ultrastructural study of FV 3 uptake

Electron micrographs of cells infected with enveloped or with naked FV 3 and fixed immediately after the 1 h adsorption period at 4 °C showed a large number of virus particles around the cells (Fig. 1), some adsorbed at the plasma membrane (Fig. 1, inset) or close to coated pits, but never inside the cytoplasm. This indicates that penetration of FV 3 does not occur at 4 °C.

Uptake of enveloped FV 3 at 29 °C

Enveloped virions were observed in coated pits (Fig. 2). They could also be seen in coated vesicles within the cell, close to the plasma membrane (Fig. 3). Later, they were found deeper in the cell, inside smooth cytoplasmic vacuoles (Fig. 4). Finally, partially degraded virus particles could be seen inside secondary lysosomes where dense degradation products were present (Fig. 5). However, these different steps were not synchronous since enveloped virions were observed close to coated pits at the end of the adsorption at + 4 °C (0 h post-infection) as well as 1 h after the transfer to 29 °C. Virus particles could be found inside cytoplasmic vacuoles after 10 min post-infection and in secondary lysosomes after 30 min post-infection whereupon their number increased as infection proceeded.

Uptake of naked FV 3 at 29 °C

Naked virus particles could sometimes be seen in coated pits (Fig. 6) or inside intracytoplasmic vesicles that were entirely or partially coated (Fig. 7). These individual vesicles appeared then to enlarge by fusing with inner cell membranes, thereby giving rise to cytoplasmic vacuoles (Fig. 8). At later times, some naked virions could also be found in secondary lysosomes similar to that shown in Fig. 5. The instances of naked FV 3 entering the cells by the same type of endocytosis as enveloped FV 3 via coated pits and coated vesicles represented only 1 to 2% of the observed interactions between FV 3 and the plasma membrane.

Observations of fusion between the viral capsid and the plasma membrane were far more frequent (the remaining 98%, Fig. 9, 10 and 11). The first step of the fusion process was characterized by a little defect in the plasma membrane which fuses with that of the virus (Fig. 9). Then the virus dense inner material was injected into the cytoplasm (Fig. 10). In both cases, the inner part of the particle displayed a less dense zone facing the cytoplasm. The last step shows the integration of the virus shell into the cell membrane (Fig. 11); the opened virus membrane appears filled with granular material of the same density as the cytoplasm. The observations of this step were very rare; afterwards, no recognizable viral structures could be observed either in the plasma membrane or in the cytoplasm.

These fusions with the plasma membrane did not seem to depend on the pH, since decreasing the extracellular pH to 5.5 had no effect on the frequency of such events.

Fig. 6 to 8. Internalization of naked FV 3 following 1 h at 4 °C and further incubation for the indicated times at 29 °C.
Fig. 6. Particle with an electron-lucent core close to a coated pit (30 min).
Fig. 7. Virion inside a vesicle, the lower part of it coated (45 min).
Fig. 8. Nucleocapsid present inside a vacuole which is being enlarged by fusion with intracellular membranes (20 min).

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Fig. 9 to 13. Fusion of naked FV 3 with cellular membrane at 29 °C. All bar markers represent 100 nm.

Fig. 9 to 11. Fusions with the plasma membrane. On Fig. 9 (1 h) the plasma membrane is cross-sectioned, whereas it is tangentially cut on Fig. 10 (20 min). In both cases, the inner part of the virion displays a light zone facing the cytoplasm. The virion on Fig. 10 seems to have injected its contents into the cell. Fig. 11 shows a viral capsid integrated into the cell membrane. No viral structure is recognizable in the cytoplasm.

Fig. 12 and 13. Fusion between naked viral particles and the membranes of intracytoplasmic vacuoles. The continuity between the lipid viral membrane underlying the capsid and the vacuolar membrane is clearly visible on Fig. 12 (fixation according to Takahashi, 1980; 1.5 h). The nucleoid is still intact. In the large intracellular vacuole on Fig. 13 (3 h) many virions can be seen. In the left part, a viral particle fuses with the vacuole membrane, and probably sheds its contents into the cytoplasm (arrow).
Within the cytoplasm, fusions were also found to occur between the membrane of intracytoplasmic vacuoles and the capsids of ingested virions (Fig. 12 and 13). In Fig. 13, several particles can be seen inside a large vacuole; the capsid of one such particle, which has fused with the vacuole membrane, seems to have shed its contents into the cytoplasm (arrow). Fusions with inner cell membranes were however less frequent than with the plasma membrane (about 17% of all the fusions observed).

As in the case of enveloped FV 3, the different steps of penetration by naked virus were not synchronous. Fusion as well as endocytosis could be observed at all times after infection.

**Effect of lysosomotropic agents on the multiplication of naked and enveloped FV 3**

The morphological observations described above indicate that lysosomes are involved in the fate of enveloped virions that penetrate into cells by endocytosis via coated pits whereas in the case of naked particles which may fuse directly with the plasma membrane, they play no role. This led us to compare the multiplication of enveloped and naked virions in cells where the lysosomal function had been blocked by chloroquine. Prior to these experiments we had demonstrated by biochemical techniques that chloroquine has no effect on FV 3 binding and did not prevent FV 3 internalization, although this process appeared reduced by 20% and 30% for enveloped and naked particles respectively. Furthermore, FV 3 suspensions incubated with chloroquine did not lose their infectivity (not shown).

In order to reduce the infectivity of naked particles which are occasionally present in enveloped FV 3 preparations (Braunwald et al., 1979), suspensions were pretreated with DNase which preferentially inactivates naked FV 3 (J. Braunwald et al., unpublished results). Under these conditions, the multiplication of enveloped virus was totally inhibited when chloroquine was added at the moment of infection (Fig. 14a, column 3), and reduced by 96.5% when the inhibitor was added 1 h later (Fig. 14a, column 4). When chloroquine was added 3 h after infection, the virus yield was greater but not as high as in the absence of the drug (Fig. 14a, column 5). Even when chloroquine was added at later times (5 h and 7 h; Fig. 14a, columns 6 and 7), the virus yield did not reach the same level as in untreated cells (Fig. 14a, column 2). The
Fig. 15 to 17. Cells infected for 18 h in the presence or absence of chloroquine. All bar markers represent 1 μm.

Fig. 15. Infection with enveloped FV 3, in the absence of chloroquine. Many viral particles can be seen, gathered into a paracrystalline array or budding at the plasma membrane.

Fig. 16. Infection with DNase-treated enveloped FV 3 suspension, in the presence of chloroquine from the beginning of the infection. No viral morphogenesis can be seen. Swollen lysosomes look like electron-lucent vacuoles. The dark spots inside them may be the nucleoids of degraded virions.

Fig. 17. Infection with naked FV 3, in the presence of chloroquine from the beginning of the infection. All signs of viral morphogenesis are present: a viroplasm (V), and mature virions either gathered into a small paracrystalline array, or budding at the plasma membrane (lower part of the cell, left and right).
multiplication of naked FV 3 (Fig. 14b) was decreased but not completely inhibited when chloroquine was added during the viral adsorption period or after 1 h of incubation at 29°C (Fig. 14b, columns 3 and 4). Data obtained with enveloped FV 3 suspensions not treated with DNase were intermediate (Fig. 14c), presumably due to the presence of some naked particles able to multiply in cells where lysosomes are impaired. Other lysosomotropic agents, such as methylamine or ammonium chloride led to similar data, although with a lower level of inhibition than chloroquine (data not given here).

Electron microscopic observations of cells infected at the same multiplicity and fixed 18 h post-infection confirmed the titration experiments. In the absence of chloroquine, many virions, clustered in paracrystalline arrays or budding at the plasma membrane (Fig. 15), were present in cells infected either by naked or enveloped FV 3. In the presence of chloroquine from the beginning of infection, cells infected with enveloped FV 3 suspensions previously incubated with DNase lacked any sign of viral multiplication (Fig. 16), while cells infected with naked FV 3 displayed a reduced number of virions (Fig. 17). However, neither gathering into paracrystalline arrays nor budding were inhibited. In chloroquine-treated cells, infected (Fig. 16 and 17) as well as uninfected (not shown), lysosomes were swollen and appeared as large vacuoles.

**DISCUSSION**

The large size of FV 3 as well as the availability of naturally occurring enveloped and naked viral particles made this virus an interesting model for the study of the early stages of interaction with susceptible host cells and for the understanding of how such viruses move from one cell to another. The present work is restricted to the first 3 h of infection to avoid the occurrence of progeny virions (Bingen-Brendel *et al.*, 1971).

After 1 h adsorption at 4°C, all the viral particles, enveloped or not, were found outside the cell, more or less close to the plasma membrane. This ultrastructural evidence that no penetration takes place at a low temperature is supported by biochemical studies of FV 3 internalization (Braunwald *et al.*, 1984). Thus, FV 3 behaves in a different way from influenza virus whose RNA moves and becomes associated with the nucleus at 4°C (Stephenson & Dimmock, 1975; Possee & Dimmock, 1981).

After the adsorption step at 4°C, FV 3-infected cells were transferred to 29°C and then penetration occurred. Enveloped FV 3 seems to be internalized via coated pits and then to move through coated vesicles, smooth vacuoles and finally lysosomes. These morphological results suggest that enveloped FV 3 is taken up by a mechanism related to receptor-mediated endocytosis, which occurs via clathrin-coated pits and vesicles (Goldstein *et al.*, 1979). Since specific cellular receptors are unknown for FV 3 any number of cell surface components may act fortuitously as binding sites for the virus; it is thus preferable to name this pathway adsorptive endocytosis, as used for uptake of Semliki Forest virus (SFV) (Marsh & Helenius, 1980).

The most frequently observed interaction between naked FV 3 and the plasma membrane is fusion which leads to the incorporation of the virus capsid into the cell membrane. The pictures of integrated capsid fragments are rare, which seems to indicate that this step is very fast. Afterwards, capsid constituents should be dispersed in the fluid cell membrane, and thus are no longer recognizable. The fate of the virus nucleoprotein content after injection by the fusion process is also an open question; indeed, no organized viral structure can be seen inside the cytoplasm in comparison to the situation with vaccinia virus where uncoated virions are well-defined structures. As for FV 3 it seems likely that viral macromolecules diffuse very quickly inside the cytosol.

Fusion of naked FV 3 with the membrane of intracytoplasmic vacuoles can also occur, although less frequently (17% of the fusions observed). In secondary lysosomes, where dense degradation products are present, no fusing particles were seen. Such fusions with lysosomal membranes might moreover require a low pH as shown for SFV (White *et al.*, 1980), vesicular stomatitis virus and influenza virus (White *et al.*, 1981). This does not seem to be the case for FV 3, since decreasing the pH of the extracellular medium did not enhance fusion with the plasma membrane. The fusion process thus seems to occur at neutral pH, at the outer cell membrane or in endocytotic vacuoles, but not in lysosomes.
This ability of a non-enveloped virus to fuse with cell membranes has already been shown for vaccinia virus (Dales, 1973; Chang & Metz, 1976; Steffan & Kirn, 1982) and for FV 3 in mouse and rat Kupffer cells in vivo (Gendrault et al., 1980) and in vitro (Gendrault et al., 1981).

Penetration of enveloped particles by adsorptive endocytosis implies a role for lysosomes in this process. This has been confirmed by study of the multiplication of both populations in the presence of chloroquine, an efficient lysosomotropic agent (De Duve et al., 1974). Inhibition of viral multiplication by lysosomotropic agents may act by inhibiting either virus delivery to lysosomes (Talbot & Vance, 1982), or the processing of viral constituents (if they reach the organelle) due to the increase of pH which inhibits the action of the hydrolases and stabilizes the lysosomal membranes (De Duve et al., 1974). In any case, lysosomotropic agents inhibit the multiplication of viruses taken up by adsorptive endocytosis, and not those internalized by fusion. Our data, both obtained by infectivity titration and electron microscopic observations, clearly show that lysosomotropic agents totally inhibit the multiplication of enveloped FV 3, when added at the very beginning of infection. It should be kept in mind that multiplication experiments and the corresponding electron microscopic controls were performed with a far lower multiplicity of infection than ultrastructural studies of internalization, and therefore caution must be observed in comparison of the two kinds of data.

The early block in FV 3 replication induced by chloroquine suggests a requirement for functional lysosomes in the first steps of viral morphogenesis, even though we do not know exactly which event is concerned. In the case of SFV, this is thought to be due to uncoating by fusion of viral particles with the lysosomal membranes (Helenius et al., 1980, 1982). Reovirus may also be found inside lysosomes, where its proteins but not its double-stranded RNA are digested (Silverstein & Dales, 1968). An uncoating process inside intracytoplasmic vesicles has already been postulated for FV 3 (Houts et al., 1974) and for another member of the Iridoviridae family, Tipula iridescent virus (Mathieson & Lee, 1981).

Among numerous reports dealing with the events related to the penetration of eukaryotic cells by viruses, some pathways seem to emerge: direct penetration, fusion and endocytosis (for reviews, see Dales, 1973, 1978; Lonberg-Holm & Philipson, 1974, 1980; Kohn, 1979; Dimmock, 1982; Bukrinskaya, 1982; Lenard & Miller, 1982; Marsh, 1984). From our study of the uptake of FV 3, it seems that the presence of a cell-derived viral envelope favours adsorptive endocytosis and not the fusion process, whereas the naked viral particles can fuse with either external or intracytoplasmic cellular membranes. This ability for fusion of naked FV 3, most likely due to the presence of an inner lipid membrane underlying the icosahedral capsid (Stoltz, 1973; Cuillel et al., 1979), is difficult to explain unless we consider the capsid as a dynamic system where spontaneous fluctuations would make the capsomeres move apart to give way to the lipid–lipid interactions required for the fusion. Such a dynamic system in the FV 3 capsid has been recently demonstrated by ultrasonic absorption (Robach et al., 1983); although it is not ascertained whether the observed fluctuations are sufficient to explain the fusion process, they may at least trigger more important motions of the capsomeres, which could allow the contact between the two membranes, viral and cellular.

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REFERENCES

Journal de Microscopie 21, 249–258.


Advances in Virus Research 27, 141–204.

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