Influenza Virus Uncoating in Infected Cells and Effect of Rimantadine

By A. G. BUKRINSKAYA,* N. K. VORKUNOVA, G. V. KORNILAYEVA, R. A. NARMANBETOVA AND G. K. VORKUNOVA

The D.I. Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences, Moscow, U.S.S.R.

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SUMMARY

Uncoating of influenza virus (strain WSN) in MDCK cells was studied by following the fate of the virus labelled with radioactive precursors. The accumulation of subviral components of input virus was observed in nuclear-associated cytoplasm (NAC) obtained by treatment of the nuclei with citric acid. Two types of subviral components were found there, ribonucleoproteins (RNPs) and larger subviral particles (SVP) containing RNPs in association with M protein. SVP, with different relative amounts of M protein, were revealed in NAC, suggesting that M protein was gradually released from RNPs. The released RNPs entered the nuclei while M protein accumulated within perinuclear membranes. Thus, SVP could be regarded as probable intermediates in virus uncoating. Rimantadine prevented the release of M protein from RNPs and their penetration into the nuclei provoking the accumulation of subviral components in NAC.

INTRODUCTION

Influenza virions, as well as other budding viruses such as paramyxo- and rhabdoviruses, possess an outer lipoprotein envelope which consists of a lipid bilayer derived from host cell plasma membrane, and glycoproteins inserted into it. Beneath the lipoprotein envelope there is a layer of matrix protein (M protein), the smallest unglycosylated virus polypeptide (mol. wt. about 25000), which is associated with RNPs (Lenard, 1978). Soon after penetration, influenza virions lose their envelopes, and the released nucleocapsids enter the cell nuclei (Hudson et al., 1978; Bukrinskaya & Vorkunova, 1979; Bukrinskaya et al., 1979). However, the events which lead to uncoating of influenza virus particles and to the release of the nucleocapsids are poorly understood. An interest in this stage of the virus replication cycle has recently arisen in connection with the concept that amantadine hydrochloride and its structural analogue rimantadine, effective inhibitors of influenza A virus replication, affect some virus-specific event after virus penetration but before primary transcription, most probably input virus uncoating (Kato & Eggers, 1969; Skehel et al., 1977; Koff & Knight, 1979).

We studied the influenza virus uncoating process by infecting cells with the viruses labelled with the radioactive precursors and following their fate in infected cells. The results seem to show that uncoating is a multistep process, and probable intermediates are subviral particles containing RNPs in association with M protein. Rimantadine interferes with the final stage of uncoating, i.e. the removal of M protein from RNPs.
METHODS

**Virus.** Influenza A virus strain WSN (H1N1) was passaged in embryonated eggs at low multiplicity (10 ID$_{50}$/egg) to avoid the accumulation of defective-interfering (DI) particles. Egg-grown virus with a p.f.u. to HA (haemagglutinin) ratio of $10^{4.3}$ to $10^{5.7}$ was used as inoculum in preparing the radioactive viruses.

**Radioactive virus.** To label the virus, confluent monolayers of chick embryo fibroblast (CEF) cells in roller bottles were infected with freshly harvested allantoic fluid diluted in saline to give an m.o.i. of approx. 10. After 1 h adsorption at 37 °C the inoculum was replaced by maintenance medium (0.5% lactalbumin hydrolysate containing 2% bovine serum for [3H]uridine labelling, and 0.5% lactalbumin hydrolysate diluted 1:10 with Hanks’ solution containing 2% bovine serum for 14C-amino acid labelling). Fresh allantoic fluid from uninfected eggs (up to 30%) was added to the medium of infected cells to cleave HA in progeny virus particles due to the effect of plasmin (Lazarowitz et al., 1973). Two h after incubation at 37 °C, [3H]uridine (100 μCi/ml) or 14C-labelled algal hydrolysate (50 μCi/ml) were added. Eighteen h after infection the culture fluid was harvested and the virus purified as described previously (Bukrinskaya & Vorkunova, 1979; Bukrinskaya et al., 1979). The specific activity of virus samples varied from 5 to 20 ct/min per 1 p.f.u.

**Infection of cells.** Confluent monolayers of CEF and MDCK (canine kidney) cells were infected with labelled virus at an m.o.i. of about 10 to 20. The adsorption was performed at 4 °C for 1 h. The inoculum was removed and the cells were washed with Hanks’ solution, brought to 37 °C by the addition of warm Eagle’s medium (this time representing zero time of infection) and incubated at 37 °C for 30 min. The monolayers were then rinsed with cold Hank’s solution and the cells harvested by scraping with glass beads.

**Treatment of cells with rimantadine.** A 25 or 50 μg/ml amount of rimantadine in warm Eagle’s medium was added to the cell monolayers 1 h before infection. Thereafter, the medium was removed, and the cells infected with the virus inoculum containing the same concentration of rimantadine. After adsorption, the inoculum was removed, the same rimantadine-containing medium which was used before infection was added, the cells were incubated for 30 min at 37 °C and then fractionated.

**Cell fractionation.** Cells were washed with reticulocyte standard buffer (RSB; 0.01 M-tris–HCl pH 7.4, 0.01 M-KCl, 0.0015 M-MgCl$_2$). After 10 min at 0 °C they were broken by 20 to 30 strokes of a tight-fitting Dounce homogenizer and the nuclei were pelleted at 800 g for 5 min. The supernatant was designated ‘cytoplasmic fraction’ and was further fractionated by centrifugation at 15,000 g for 15 min. The supernatant (S$_{15}$) was designated ‘cytoplasmic extract’. The nuclei were resuspended in 0.1 M-citric acid (Ro-Choi et al., 1973) containing 0.001 M-MgCl$_2$, and the mixture vortexed for 5 min at 4 °C and immediately neutralized with tris buffer pH 8.8. Thereafter, the nuclei were pelleted at 1000 g, and the supernatant was removed and designated ‘nuclear-associated cytoplasm’ (NAC). The nuclei were treated with 1% Triton X-100. Nucleoplasm (nuclear extract) was obtained as described previously (Bukrinskaya & Vorkunova, 1979; Bukrinskaya et al., 1979).

**Sedimentation and buoyant density analysis.** The centrifugation in 15 to 40% velocity glycerol gradients and in CsCl density gradients was performed as described previously (Bukrinskaya & Vorkunova, 1979; Bukrinskaya et al., 1979).

**Cell autoradiography.** MDCK cells grown on coverslips in Leiton tubes were infected with [3H]uridine-labelled virus (1 × 10$^5$ to 3 × 10$^5$ ct/min/coverslip). The adsorption was performed for 1 h at +4 °C, and the inoculum then removed and the cells thoroughly washed with cold phosphate-buffered saline (PBS). Warm Eagle’s medium was added, and the cells were transferred to 37 °C, this time representing zero time. Thirty min after infection the cells were washed with Hank’s solution, fixed, dipped in photographic emulsion and stored at +4 °C for autoradiographic exposure. After 2 to 3 weeks the films were developed and specimens were stained.
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SDS–polyacrylamide gel electrophoresis (SDS–PAGE). This was carried out according to Laemmli (1970) using 10% acrylamide gels. After staining and destaining to visualize standard proteins, the gels were dried and processed for autoradiography or fluorography.

Chemicals and isotopes. All chemicals used for PAGE were purchased from Bio-Rad, [3H]uridine (sp. act. 25 Ci/mmol) from Radiochemical Centre, Leningrad, U.S.S.R., and 14C-labelled algal hydrolysate (1 mCi/mg) from Prague, Czechoslovakia. Rimantadine hydrochloride was obtained from the Institute of Organic Synthesis, Latvian Academy of Sciences, U.S.S.R.

RESULTS

Distribution of input virus radioactivity in subcellular fractions and the properties of subviral components

CEF and MDCK cells were infected with influenza WSN virus labelled with 14C-amino acids, fractionated 30 min after incubation at 37 °C, and acid-insoluble radioactivity was determined in subcellular fractions. A significant part of parental virus radioactivity was found in the cytoplasmic fraction and the bulk of this radioactivity was revealed in the pellet after centrifugation at 15 000 g for 10 min (P15 fraction). When the proteins of the pellet were analysed by SDS–PAGE, the polypeptide patterns were identical to that of the virus (not shown), indicating that the radioactivity of this material was most probably due to the virus adsorbed on plasma membrane or penetrated into the cell within endocytic vacuoles (Dourmashkin & Tyrrell, 1974). The rest of the radioactivity was distributed between nuclear-associated cytoplasm (NAC) and nuclei (about 40% and 60% respectively).

The radioactive components in subcellular fractions were analysed by centrifugation in velocity glycerol gradients and density CsCl gradients, and their proteins were identified by SDS–PAGE. The radioactive components in NAC obtained from WSN-infected MDCK cells sedimented in two regions of glycerol gradients, at about 80S and 50S, and significant radioactivity was observed at the top of the gradient (Fig. 1 a). Meanwhile, the radioactive structures in the nucleoplasm of the same cells sedimented at 50S (Fig. 1 b). The 80S component in NAC, when recentrifuged in a CsCl gradient, banded at 1.28 to 1.32 g/ml, i.e. at the density intermediate between that of the virus and RNPs. The 50S component in NAC banded at 1.35 g/ml, the density expected for virus RNPs (Fig. 1 c, d).

When the proteins of the 50S component pelleted from the glycerol gradient were analysed by SDS–PAGE, the single polypeptide NP was revealed in autoradiographs, suggesting that this structure represented the parental RNPs. The minor P proteins were not detected due either to their insignificant amounts, or to their removal from the parental RNPs during the uncoating process or purification procedure (see Fig. 2 b).

The 80S component pelleted from glycerol or CsCl (without formaldehyde) gradient fractions was shown to contain two polypeptides, NP and M. The relative amounts of these polypeptides varied, generally reflecting the position of the particles in glycerol and CsCl gradients. As shown in Fig. 2, the ratio of M to NP polypeptides was reduced as the density of the particles increased from 1.28 to 1.32 g/ml. These results are consistent with the suggestion that the 80S subviral particles (SVP) contain RNPs associated with M protein, and M protein could be gradually removed from RNPs. Accordingly, electron microscopic observation of the 80S component pelleted from NAC revealed the presence of round particles with helical strands inside (Fig. 3). The particles of lower density contained traces of HA, suggesting the presence of some associated lipid.

As shown in Fig. 4, the high radioactivity was consistently found on the top of the glycerol gradients after centrifugation of NAC from infected cells. The sensitivity of the top material to detergent (Nonidet P40) and its low buoyant density (1.18 to 1.20 g/ml in CsCl) (not shown) suggest its membrane origin. When the proteins of the top components were analysed
Fig. 1. Characteristics of influenza virus WSN parental subviral particles in the nuclear-associated cytoplasm and nucleoplasm of MDCK cells. MDCK cells infected with $^{14}$C-labelled WSN were fractionated 30 min after infection. (a) NAC and (b) nucleoplasm were centrifuged in a 15 to 40% glycerol gradient prepared on TNE buffer (0.01 M-tris–HCl pH 7.4, 0.1 M-NaCl, 0.001 M-EDTA), in a Spinco SW41 rotor at 14000 rev/min for 16 h at 4 °C. The arrows here and in the other figures show the position of 50S ribosomal subunit centrifuged in parallel tubes and revealed by absorbance. Aliquots of gradient fractions in (a) were processed for acid-insoluble radioactive determination; the peak fractions were diluted 1:1 with PBS containing 0.001 M-EDTA and formaldehyde at a 4% final concentration, and recentrifuged in a preformed CsCl gradient prepared in the same buffer in an SW50 rotor at 35000 rev/min for 16 h at 4 °C and processed for acid-insoluble radioactive determination. (c, d) 80S and 50S components respectively.

by SDS–PAGE, two polypeptides, NP and M, were revealed. To differentiate between the proteins of subviral components associated with perinuclear membranes and the free proteins integrated into the membranes, the top material was treated with 1% Nonidet P40, and centrifuged for 2 h at 150000 g to pellet the subviral particles. The proteins of the pellet and supernatant were then analysed by SDS–PAGE. NP and M protein were found in the pellets while predominantly M protein was revealed in the supernatant (Fig. 4). The finding that high concentrations of a single M protein are accumulated in perinuclear membranes supports the view that this protein could dissociate from RNPs in the process of uncoating, and this step occurs in close proximity to the nucleus. The results obtained with WSN-infected chicken fibroblasts were very similar to those obtained with WSN-infected MDCK cells, suggesting that the uncoating process described was universal for different host cells.
Fig. 2. Polypeptides of influenza virus WSN subviral particles of different densities. MDCK cells were infected with ^14^C-labelled WSN. NAC obtained 30 min after infection was centrifuged in a CaCl gradient prepared on PBS containing 0.001 M-EDTA but without formaldehyde. The radioactive components were pelleted from each gradient fraction and their proteins were separated on 10% gels and processed for autoradiography. (a) WSN virus used as a marker; (b) RNPs; (c to f) SVP of densities (g/ml) of (c) 1.32, (d) 1.30, (e) 1.29, and (f) 1.28.

Effect of rimantadine on virus uncoating

When added 30 min before infection, rimantadine treatment caused a 15-fold reduction in the infectious titre of progeny virus. Table 1 shows the distribution of parental virus radioactivity in cells untreated and treated with rimantadine. As mentioned above, the radioactivity of the P_{15} fraction was most probably due to virus particles adsorbed on the plasma membrane and was therefore not taken into account when the percentage of intracellular radioactivity was counted. As seen from the table, the relative amounts of radioactivity in cytoplasmic extracts and Triton washings were similar in untreated and
Fig. 3. Electron micrographs of the 1.29 g/ml material from peak fractions of the gradient similar to that shown in Fig. 1 (c). Negative staining with uranyl acetate (×380000).

Fig. 4. Polypeptides of top components in NAC. Cells were infected with 14C-labelled WSN virus and NAC was centrifuged in a glycerol gradient as described in Fig. 1 (a). Top components from Nonidet P40-treated top material were pelleted from the corresponding pooled fractions by centrifugation at 15 000 g for 2 h. The proteins of the supernatant were precipitated with 3 vol. acetone. The proteins of (a) the supernatant and (b) the pellet were separated on a 10% gel and processed for autoradiography.
Fig. 5. Effect of rimantadine on virus uncoating. Untreated and rimantadine-treated MDCK cells were infected with 14C-labelled WSN virus; NAC and nuclear extracts were obtained and analysed in CsCl gradients as described in Fig. 1. (a) NAC from untreated (○) and rimantadine-treated (●) cells; (b) nuclear extract from untreated (○) and rimantadine-treated (●) cells; (c) polypeptides of WSN virus (lanes 1 and 2), of 1.30 to 1.31 g/ml (lane 3) and 1.28 to 1.29 g/ml (lane 4) components isolated from rimantadine-treated cells and analysed by SDS-PAGE followed by autoradiography as in Fig. 2.

Table 1. Effect of rimantadine on the distribution of input virus radioactivity among subcellular fractions*

<table>
<thead>
<tr>
<th>Subcellular fraction</th>
<th>Control</th>
<th>Rimantadine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ct/min × 10^3</td>
<td>%</td>
</tr>
<tr>
<td>P15 fraction</td>
<td>159.4</td>
<td>–</td>
</tr>
<tr>
<td>Cytoplasmic extract</td>
<td>36.0</td>
<td>7.4</td>
</tr>
<tr>
<td>Triton washings</td>
<td>82.8</td>
<td>16.9</td>
</tr>
<tr>
<td>NAC</td>
<td>151.0</td>
<td>30.9</td>
</tr>
<tr>
<td>Nuclei</td>
<td>219.1</td>
<td>44.8</td>
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* MDCK cells were treated with rimantadine (50 μg/ml) for 1 h at 37 °C; thereafter, the medium was removed, the cells were infected with WSN virus labelled with 14C-amino acids (sp. act. 2 × 10^4 to 5 × 10^4 ct/min/HA unit). The same rimantadine-containing medium was added, and the cells were incubated for 30 min at 37 °C. Thereafter, the cells were fractionated, and the acid-insoluble radioactivity in subcellular fractions determined.

rimantadine-treated cells. By contrast, the relative amounts of radioactivity in NAC and nuclei in rimantadine-treated cells were significantly altered: the radioactivity was increased in NAC and decreased in nuclei.

Predominantly SVP were accumulated in NAC of WSN-infected rimantadine-treated cells. They sedimented at 80S to 100S in glycerol gradients, banded at 1.28 to 1.30 g/ml in CsCl gradients (Fig. 5a) and contained NP and M proteins as revealed by SDS–PAGE analysis (Fig. 5c). Few RNPs of the density 1.35 g/ml and relatively large amounts of SVP were extracted from the nuclei of rimantadine-treated cells (Fig. 5b). The component of higher density (1.39 to 1.41 g/ml), presumably modified RNPs (Bukrinskaya & Vorkunova, 1979; Bukrinskaya et al., 1979), was never found in the nuclei of rimantadine-
Fig. 6. Autoradiographs of MDCK cells 30 min after infection with [3H]uridine-labelled virus. 
(a) Cells treated with a mixture of WSN virus and WSN antiserum; (b, c) WSN virus-infected cells; 
(d to g) rimantadine-treated infected cells.
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The origin of the 'nuclear' SVP is not clear; most probably they represent extranuclear particles which still remain in association with nuclei after citric acid treatment.

The redistribution of intracellular virus structures in rimantadine-treated cells was also demonstrated using the method of cell autoradiography. MDCK cells untreated and treated with rimantadine were infected with [³H]uridine-labelled WSN virus and, 30 min after infection, processed for autoradiography. As shown in Fig. 6(b, c), most of the grains in untreated cells are located above the nuclei, being randomly distributed over the nuclear territory or associated with the nucleoli (see Fig. 6c). Much smaller numbers of grains could be seen over the cytoplasm. To show that the radioactive grains do correspond to the virus structures, the virus inoculum was mixed with antiserum to WSN with a neutralization titre of 1:512, diluted 1:10; the mixture was incubated at room temperature for 30 min and then added to the cells. No grains over the cells were observed in this case (Fig. 6a).

When rimantadine-treated cells were infected with ³H-labelled WSN virus, a striking difference in grain distribution was observed as compared to that in untreated cells. The nuclei were practically free of grains, the bulk of them being accumulated in poorly stained vacuolized regions of the cytoplasm to one side of the nucleus (Fig. 6d). In some autoradiographs this region was enlarged and differentiated from the rest of the cytoplasm (Fig. 6e, g). The chain of grains along the nuclear membrane was distinctly seen in a number of cells (Fig. 6f). These data support the suggestion that rimantadine blocks the transport of parental virus structures to the nuclei.

Taking into account the results obtained by cell fractionation, it could be suggested that the poorly stained region of the cytoplasm in close proximity to the nucleus corresponds to the 'nuclear-associated cytoplasm' in cell fractionation experiments.

**Discussion**

Our data demonstrate that early in infection two types of parental virus components are found in infected cells. One consists of typical RNPs while the other represents the larger subviral component of buoyant density intermediate between that of the virus and RNPs (1.28 to 1.32 g/ml). Two proteins, NP and M, have been found within these subviral particles (SVP), suggesting that they contain RNPs associated with M protein. In this respect, SVP are similar to virus cores obtained by the treatment of influenza virus with detergents (Schulze, 1972). In SVP with the lowest density (1.28 g/ml) traces of HA were detected. It follows that these particles could contain some associated virus lipids.

The gradual decrease in relative amounts of M protein, proportional to the increase in buoyant density of the particles, and accumulation of free M protein in perinuclear membranes supports the idea that M protein could gradually dissociate from the surface of SVP and the final products are the 1.35 g/ml RNPs. From this point of view, SVP could be regarded as intermediates in virus uncoating. They are accumulated in close proximity to the nucleus, and apparently are not free but associated with perinuclear endoplasmic reticulum or with nuclear membrane. It is not clear why citric acid, which is widely used for purification of nuclei (see for example Ro-Choi et al., 1973), extracts membrane-associated parental SVP and RNPs. This effect could be due to the profound damage of perinuclear membranes or outer nuclear membrane by citric acid. The final event of uncoating, the release of RNPs, occurs in intimate association with perinuclear or nuclear membrane or between outer and inner nuclear membranes within perinuclear cisternae, and the released RNPs enter the nuclei.

The possibility could not be eliminated that citric acid produces some effect on the intracellular virus particles, giving rise to the artificial subviral components observed in our experiments. This seems unlikely, however, since brief treatment of intact WSN virus with
citric acid has been shown not to disrupt the virions, all the radioactivity being pelleted after centrifugation through 15 to 40% glycerol gradients at 35000 rev/min for 2.5 h. In addition, HA₁ and HA₂ polypeptides were never revealed in NAC material. If our interpretation is true and SVP are not artefacts of citric acid treatment, then the uncoating process seems to proceed in two steps: (i) removal of the outer lipoprotein membrane with embedded glycoproteins and the release of SVP; (ii) removal of the M protein and the release of RNPs. As shown, these two steps proceed in different cell compartments. Intact virions and possibly virions at the beginning of uncoating were found in the cytoplasm in association with cellular membranes, while SVP and RNPs were revealed in NAC. So far, the following hypothetical scheme of influenza virus uncoating could be proposed. The virus particles penetrate cells by fusion with the plasma membrane (Morgan & Rose, 1968; Huang et al., 1980) or by endocytosis (Dourmashekin & Tyrrell, 1974) followed by fusion of outer virus lipoprotein membrane with the membrane of endocytic vacuoles (Huang et al., 1980). As a result of fusion, SVP are liberated into the cytoplasmic matrix, or remain associated with vacuole membranes; both free and phagosome-associated subviral particles were distinctly seen in electron micrographs by Dourmashekin & Tyrrell (1974). SVP are transported to the nuclear membrane apparently along the membranes of endoplasmic reticulum; their association with membranes agrees well with the high hydrophobic properties of M protein. At the nuclear membrane the second step of uncoating, the release of RNPs from M protein, occurs, possibly by fusion of the M protein with perinuclear or nuclear membrane(s). As a result of such fusion, M protein becomes incorporated into perinuclear membranes, and the resultant naked RNPs are released free into the nuclei. Such a scheme of uncoating may explain why ribonuclease-sensitive virus RNPs could reach the nuclei of infected cells and initiate infection.

According to this model, both steps of uncoating proceed by fusion of virus membrane with cellular membranes and thus do not require the participation of cell enzymes. This correlates well with the findings (Stephenson & Dimmock, 1975; Hudson et al., 1978) that input influenza virus RNPs reach the nuclei of infected cells at 4 °C, and with our unpublished data that both steps of uncoating could occur at 4 °C.

Rimantadine provokes a dramatic perturbation of the uncoating process, preventing the removal of M protein from RNPs. RNPs bound to M proteins apparently cannot penetrate the nuclei and initiate transcription there, thus blocking the nuclear phase of virus reproduction. Amantadine produced the same effect on uncoating as rimantadine (A. G. Bukrinskaya et al., unpublished results).

In rimantadine-treated cells subviral particles are accumulated in nuclear-associated cytoplasm. The poor staining and high vacuolization of this region of the cytoplasm suggest the involvement of specific cellular organelles, possibly special endoplasmic reticulum adjacent to the Golgi complex and to the nucleus and containing lysosomes (GERL; Novikoff & Novikoff, 1977). Such localization seems to agree with the data of Helenius et al. (1980) that intralysosomal events are responsible for the antiviral effect of amantadine.

The mechanism which prevents the removal of M protein from RNPs in amantadine- and rimantadine-treated cells is unknown. In vitro experiments showed that the drugs prevented binding to liposomes of M protein associated with SVP or isolated from purified virions (A. G. Bukrinskaya et al., unpublished results). These results are in accordance with the data that the gene for M protein is responsible for the sensitivity of influenza viruses to the drug (Lubeck et al., 1978; Hay et al., 1979). In this respect, of great interest is the uncoating of influenza virus strains resistant to rimantadine, the data described in the following communication (Bukrinskaya et al., 1982).
REFERENCES


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