Uncoating of a Rimantadine-resistant Variant of Influenza Virus in the Presence of Rimantadine

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(Accepted 7 December 1981)

SUMMARY

A rimantadine-resistant variant of the Texas strain of influenza virus (Tr) was obtained by serial passages in eggs and in MDCK cells in the presence of the drug, and its uncoating in MDCK cells was compared to that of the sensitive variant (Ts). First and second steps of uncoating were defined respectively by the appearance of subviral particles (SVP) in nuclear-associated cytoplasm (NAC) and ribonucleoproteins (RNPs) in nucleoplasm. In cells infected with Ts, SVP and RNPs were revealed in NAC, while in the presence of rimantadine RNPs were neither found in NAC nor in the nucleoplasm. In cells infected with Tr, SVP but not RNPs were observed in NAC. The amount of RNPs in the nucleoplasm was almost unchanged in rimantadine-treated cells, demonstrating that rimantadine did not interfere with uncoating of the resistant variant. These findings confirm the suggestion that rimantadine blocks the second step of uncoating of sensitive influenza viruses, and are consistent with the idea that this event does account for the prevention of influenza virus infection by the drug.

INTRODUCTION

In the previous communication (Bukrinskaya et al., 1982) the effect of rimantadine on uncoating of influenza virus strain WSN has been described. Our findings suggest that uncoating has two steps: (i) the removal of the outer lipoprotein envelope and (ii) the removal of the matrix (M) protein and the release of RNPs. Rimantadine prevents uncoating, making impossible the second step of uncoating.

If these events account for the inhibition of influenza virus reproduction by rimantadine, the drug should not interfere with the second step of uncoating of rimantadine-resistant variants of influenza viruses. In this paper, the uncoating of a rimantadine-resistant variant of Texas virus was compared to that of rimantadine-sensitive virus in cells untreated and treated with rimantadine. The results demonstrate that rimantadine does not block the release of RNPs of the resistant variant.

METHODS

Virus. The Texas strain of influenza A virus (H3N2) was propagated in chick embryos at low multiplicity (10 ID$_{50}$/egg). To obtain the rimantadine-resistant variant, the virus was initially passaged five times in chick embryos at a multiplicity of 6 ID$_{50}$/egg in the presence of increasing concentrations of rimantadine from 1.5 to 3 mg/egg. Then, four passages in MDCK cells in the presence of 35 to 40 μg/ml rimantadine were made. The input rimantadine-sensitive strain was passaged similarly but without rimantadine. The resistance to rimantadine was checked in monolayers of chick embryo fibroblasts by the plaque assay.
method, the agar overlayer containing 2 μg/ml trypsin (Worthington Biochemicals), 0.6% sucrose and rimantadine at concentrations from 5 to 15 μg/ml, and by intracellular haemagglutinin (HA) production.

Both sensitive (Ts) and resistant (Tr) variants were labelled in confluent monolayers of MDCK cells by [3H]uridine or 14C-algal hydrolysate (sp. act. 1 × 10^4 to 2 × 10^4 and 3 × 10^4 to 5 × 10^4 per 1 HAU respectively) and purified as described for WSN virus (Bukrinskaya et al., 1982). In some experiments, 1 μg/ml trypsin was added to the culture fluid at zero time of infection to cleave the HA precursor in virus progeny.

Experimental procedure. MDCK cells were infected in parallel with Ts and Tr at 4 °C, incubated for 30 min at 37 °C, fractionated, and subviral components in cellular fractions identified by centrifugation in velocity glycerol gradients and CsCl density gradients and by analysis of the proteins by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). All experimental details are given in the preceding paper (Bukrinskaya et al., 1982).

RESULTS

Effect of rimantadine on the reproduction of Ts and Tr

Fig. 1 (a) shows that rimantadine at concentrations from 5 to 15 μg/ml effectively inhibited plaque formation of the sensitive variant but did not interfere with that of the resistant variant. The production of intracellular HA of Ts was prevented by the low concentrations of rimantadine which were ineffective against HA of Tr. In the latter case, HA production was not inhibited by the concentrations of the drug up to 25 μg/ml although it was somewhat inhibited by 50 μg/ml. Therefore, 25 μg/ml was used in all subsequent experiments.

Distribution of radioactivity in infected cells and biophysical characteristics of subviral components

MDCK cells were infected with Ts and Tr labelled with radioactive precursors and fractionated 30 min after infection. Table 1 shows the distribution of input virus radioactivity in untreated and rimantadine-treated cells. It can be seen that rimantadine produced a different effect on the distribution of radioactivity in cells infected with Ts and Tr: in Ts-infected cells rimantadine provoked an increase of radioactivity in NAC and a decrease of that in nuclei. In contrast, in rimantadine-treated Tr-infected cells the radioactivity in NAC was not significantly changed and that in nuclei was even slightly increased.

Fig. 2 shows the sedimentation properties of the subviral components isolated from untreated and rimantadine-treated cells. Two radioactive peaks that sedimented at 50S (presumably RNPs) and near the bottom of the gradient were revealed in NAC of Ts-infected cells. The same two components, although sedimenting more heterogeneously, were observed in the nucleoplasm (Fig. 2a, b). On the other hand, no 50S component was revealed in NAC and nucleoplasm of rimantadine-treated cells (Fig. 2c, d). The heterogeneous component sedimenting at 80S to 100S was found in NAC of Tr-infected cells while the nucleoplasm again contained both 50S and rapidly sedimenting components (Fig. 2e, f). In contrast to the nucleoplasm of Ts-infected cells, the 50S component was consistently observed in the corresponding material of Tr-infected cells (Fig. 2h).

Fig. 3 shows the buoyant density of the parental virus components as determined by centrifugation in CsCl gradients. The 50S component banded at 1.35 g/ml (Fig. 3a), confirming the suggestion that it represents parental virus RNPs. The rapidly sedimenting component found near the bottom of the glycerol gradients shown in Fig. 2(a) banded at 1.25 to 1.28 g/ml while the component sedimenting in the region of 80S to 90S banded more homogeneously at 1.28 g/ml (Fig. 3b, c). Both the 1.35 and 1.28 g/ml components in similar amounts were revealed when NAC from Ts-infected cells was centrifuged in a CsCl gradient.
Rimantadine-resistant influenza variant

Fig. 1. Effect of rimantadine on the reproduction of rimantadine-sensitive and resistant variants of Texas virus. The multiplicity of infection was about 10 ID_{50}/cell. (a) Plaque formation in the presence of different concentrations of rimantadine. (b) Amount of intracellular HA 6 h after infection of chick embryo fibroblasts in the presence of different concentrations of rimantadine. ○, Sensitive variant; ●, resistant variant.

Table 1. Distribution of input virus radioactivity in subcellular fractions of Ts- and Tr-infected cells and the effect of rimantadine

<table>
<thead>
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<th>Influenza virus variant</th>
<th>Ts</th>
<th>Tr</th>
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<tr>
<td></td>
<td>Control</td>
<td>Rimantadine</td>
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<tr>
<td><strong>Fraction</strong></td>
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<td>× 10^{-2}</td>
<td>× 10^{-2}</td>
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<tr>
<td></td>
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<tr>
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<td></td>
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* MDCK cells were treated with rimantadine (25 μg/ml) for 1 h at 37 °C. Thereafter, the medium was removed, the cells were infected with ^14C-labelled viruses, the same rimantadine-containing medium was added, and the cells were incubated for 30 min at 37 °C and then fractionated. The acid-insoluble radioactivity in subcellular fractions was determined.

They were both sensitive to ribonuclease, although the 1.28 g/ml component was not fully destroyed by the enzyme (Fig. 3 d).

When the infection was performed in the presence of rimantadine, a single 1.28 g/ml component was revealed in NAC and in the nuclear extract of Ts-infected cells (Fig. 3 e, g, h), confirming our previous data obtained with WSN virus that rimantadine blocks the release of RNPs in infected cells (Bukrinskaya et al., 1982).

A different situation was observed in the cells infected with the resistant variant. The bulk of radioactivity in NAC banded heterogeneously at 1.25 to 1.30 g/ml and only a small portion was found at 1.35 g/ml. The distribution of the radioactivity was not essentially changed when the cells were treated with rimantadine (Fig. 3 f). Fig. 3 (g, h) shows the comparative analysis in a CsCl gradient of NAC and nucleoplasm from Ts- and Tr-infected rimantadine-treated cells. It can be seen that rimantadine fully eliminates the 1.35 g/ml component from Ts-infected cells but does not prevent its appearance in Tr-infected cells.

_Virus proteins_

Fig. 4 shows the proteins of the sensitive and resistant variants and of their intracellular components as revealed by SDS–PAGE followed by autoradiography. No differences were
Fig. 2. Effect of rimantadine on sedimentation properties of intracellular parental subviral components of Texas virus. MDCK cells, untreated (a, b, e, f) and treated with rimantadine (25 µg/ml) added 1 h before infection, during adsorption and after adsorption (c, d, g, h) were infected with Texas virus (a to d) and its rimantadine-resistant variant (e to h) labelled with 14C-amino acids. Thirty min after infection the cells were fractionated, nuclear-associated cytoplasm (a, c, e, g) and nuclear extracts (b, d, f, h) were obtained and centrifuged in a 15 to 40% glycerol gradient prepared in 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 show the position of 50S ribosomal subunit and 80S monosomes (in the last case EDTA was omitted from the buffer) centrifuged in parallel tubes and revealed by absorbance.

revealed in polypeptide patterns of the sensitive and resistant variants of Texas virus (lanes 1 and 2 respectively). A single NP was found in the 50S component isolated from Ts- and Tr-infected cells (lanes 3 and 4 respectively) while two proteins, NP and M, were seen in the 80S to 100S components isolated from the same cells as well as from infected rimantadine-treated cells (lanes 5 and 8 to 10). The double band in the position of NP clearly seen in the virions and in subviral components apparently corresponds to two forms of NP (Bukrinskaya et al., 1979), the modified form NP2 being 3000 daltons shorter than the main form of NP (NP1) (Zhirnov & Bukrinskaya, 1981).

Lanes 6 and 7 show respectively the proteins of Ts and Tr associated with NAC membrane fragments which are found on the top of the glycerol gradients. M protein and trace amounts of NP were seen, suggesting that M protein liberated from subviral particles is accumulated in the perinuclear membranes.

DISCUSSION

Our data show that two subviral components could be found in the nuclear-associated cytoplasm and in the nucleoplasm of MDCK cells infected with Texas virus. One of them sediments at 50S, bands in CsCl at 1.35 g/ml and contains a single protein, NP (the absence of P proteins is possibly explained by insufficient radioactivity of the samples), and thus could be identified as virus RNPs. The other component sediments more rapidly (at 80S to 100S), bands at lower densities (1.27 to 1.30 g/ml), and contains, besides NP, the other major structural protein, M, the properties making it likely that these subviral particles are virus cores. Thus, intracellular subviral components of Texas virus are identical to those of WSN virus (Bukrinskaya et al., 1982).
Fig. 3. Buoyant density in CsCl of intracellular parental subviral components of Texas virus. (a to c) Recentrifugation from glycerol gradients shown in Fig. 2 (a, c) of (a) 50S component, (b) 80S component and (c) >80S component. The material of glycerol gradient fractions was twice diluted with TNE buffer and, after fixation with formaldehyde (final concentration 4%), centrifuged in a preformed CsCl gradient prepared in phosphate-buffered saline (PBS) in a SW41 rotor at 35,000 rev/min for 16 h at 4 °C. The acid-insoluble radioactivity in gradient fractions was determined. (d) Effect of ribonuclease on RNPs and SVP. NAC from Ts-infected cells was divided in two parts: one part was made 0.001 M with respect to EDTA; the other part was treated with ribonuclease (Koch-Light; 50 μg/ml for 30 min at room temperature). It was then made 0.001 M with respect to EDTA. After fixation with formaldehyde (final concentration 4%) both samples were centrifuged in CsCl gradients in parallel tubes. ●, Ts; ○, Ts + RNase. (e, f) Effect of rimantadine on subviral components in (e) Ts-infected and (f) Tr-infected cells. NAC was obtained from untreated (○) and rimantadine-treated (●) cells and centrifuged in parallel tubes. (g, h) Subviral components in (g) NAC and (h) nuclear extracts of Ts-infected and Tr-infected cells treated with rimantadine. The material from Ts-infected (●) and Tr-infected (○) cells was centrifuged in parallel tubes.
Fig. 4. Polypeptides of intracellular subviral components. MDCK cells were infected with Ts and Tr viruses labelled with $^{14}$C-amino acids. NAC and nuclear extracts were obtained 30 min after infection, centrifuged in 15 to 40% glycerol gradients as described in Fig. 2, the 80S and 50S components and the components at the top of the gradients were pelleted at 150000 g for 2 h, and the proteins of pelleted components were processed for SDS–PAGE followed by autoradiography. Lanes 1 and 2, Ts and Tr viruses respectively; lane 3, 50S component from NAC of Ts-infected cells; lane 4, 50S component from nuclear extract of Tr-infected cells; lane 5, 80S component from NAC of Tr-infected, rimantadine-treated cells; lane 6, top component from Ts-infected, rimantadine-treated cells; lane 7, top component from Tr-infected, rimantadine-treated cells; lane 8, 80S component from NAC of Ts-infected, rimantadine-treated cells; lane 9, 80S component from NAC of Ts-infected cells; lane 10, 80S component from NAC of Tr-infected cells.

Since M protein is known to be responsible for the resistance of virus strains to amantadine (Lubeck et al., 1978; Hay et al., 1979) the variations in uncoating of sensitive and resistant variants described here could be due either to the mode of association of M protein with RNPs or to the ability of M protein to fuse with host cell membranes. In this respect, the spatial relationship of subviral particles to the host cell components during the second step of uncoating seems to be important.

The results obtained with the rimantadine-resistant variant strongly suggest that rimantadine does not interfere with the second step of uncoating of this variant and thus support the view that the blockage of this step accounts for the inhibition of influenza virus reproduction by rimantadine.

REFERENCES


(Received 20 July 1981)