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Rimantadine Hydrochloride Blocks the Second Step of Influenza Virus Uncoating

Brief Report

By

A. G. Bukrinskaya, N. K. Vorkunova, and R. A. Narmanbetova

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

With 4 Figures

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Summary

Two steps of influenza virus A/WSN uncoating in MDCK cells are described. The products of the first step are cores which accumulate in the nuclear-associated cytoplasm. The products of the second step are ribonucleoproteins which penetrate the nuclei. Rimantadine blocks the second step without interfering with the first one.

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Amantadine hydrochloride and its structural analog rimantadine hydrochloride are effective inhibitors of influenza A virus replication. However, the precise mechanism of their effect on virus reproduction is unknown. The generally agreed conclusions are that the drugs affect some initial step in the virus replication cycle, most probably input virus uncoating (6). In accordance, it has been shown recently that rimantadine interferes with WSN virus uncoating in MDCK cells inhibiting the reduction of RNase-resistant material (3).

We studied the uncoating process in WSN infected MDCK cells and the effect of rimantadine using two methodical approaches: 1. autoradiography of cells infected with WSN virus prelabeled with ^{3}H-uridine; 2. fractionation of cells infected with WSN virus prelabeled with ^{14}C-amino acids and analysis of subviral components and their proteins in cellular fractions.

Labeling of the virus in chicken fibroblasts, its purification, infection and fractionation of MDCK cells, analysis of virus-specific structures by centrifugation in glycerol and CsCl gradients and of their proteins by PAGE have been described (1). Briefly, after an 1-hour adsorption period at 4° C the monolayers were rinsed with warm Eagle's minimum essential medium (this time representing 0 time of infection), incubated at 37° C for 30 minutes, and then fractionated. One additional step was introduced into the fractionation procedure: the nuclei

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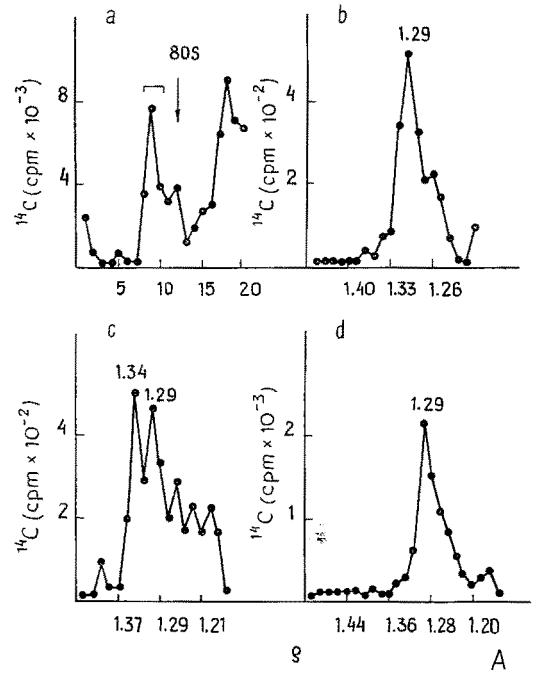
after first purification by 1 per cent Triton X-100, were resuspended in 0.1 M citric acid containing 0.001 M $MgCl_2$, the mixture was vortexed, and immediately neutralized with 1 M NaOH. Thereafter the nuclei were pelleted at $1000 \times g$, and the supernatant was removed and designated "nuclear-associated cytoplasm". The possibility that nuclear-associated cytoplasm contained virus particles (they could be adsorbed on cellular membranes cosedimented with nuclei) disrupted by such treatment was eliminated by control experiments which showed that brief contact with 0.1 M citric acid did not break the virus particle. The nuclei were further purified by centrifugation through 30 per cent sucrose, and the were further purified by centrifugation through 30 per cent sucrose, and the nuclear extract was obtained as described (1).

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

Subcellular fractions	Experiment 1				Experiment 2			
	Control		Rimantadine		Control		Rimantadine	
	cpm	%	cpm	%	cpm	%	cpm	%
Cytoplasm	203,800	—	242,000	—	159,400	—	189,400	—
Triton X-100 washings	98,200	—	148,600	—	82,800	—	103,600	—
Cytoplasmic extract (S_{20})	—	—	—	—	36,000	8.9	29,200	5.9
Nucleus-associated cytoplasm	166,400	44.7	436,600	82.5	151,000	37.1	376,200	76.7
Nuclei	206,000	55.3	92,600	17.5	219,100	54.0	85,400	17.4
Total radioactivity	674,400	—	619,800	—	612,300	—	654,600	—
Nuclear extract	70,800	34.3	66,800	72.1	46,800	32.4	62,800	71.6
Nuclear pellet	135,200	65.7	25,800	27.9	97,400	67.2	24,900	28.4

MDCK cells were treated with 50 μg/ml of rimantadine hydrochloride in Eagle's medium for one hour at 37° C, thereafter the medium was removed, the cells were infected with WSN virus labeled with ^{14}C-amino acids (sp. act. $2—5 \times 10^4$ cpm/HA unit). The same rimantadine-containing medium was added, and the cells were incubated for 30 minutes at 37° C. Thereafter the cells were fractionated, and the acid-insoluble radioactivity in subcellular fractions was determined

Fig. 1. Sedimentation and density analysis of parental virus structures in nuclear-associated cytoplasm *(A)* and nuclear extract *(B)*. MDCK cells were fractionated 30 minutes after infection with WSN virus prelabeled with ^{14}C-amino acids. *a*, *b* Nuclear-associated cytoplasm (A) and nuclear extract (B) were centrifuged in linear 15 to 30 per cent glycerol gradients prepared in TNE buffer (0.01 M Tris-HCl pH 7.4, 0.1 M NaCl, 0.001 M EDTA) in a Spinco SW 41 bucket rotor at 38,000 rpm for 3 hours (A), or at 15,000 rpm for 16 hours (B) at 4° C (a). The acid insoluble radioactivity was counted in aliquots, and the peak fractions (bracketed) were recentrifuged in preformed CsCl gradients prepared on phosphate buffer pH 7.4 containing 0.001 M EDTA and 4 per cent formaldehyde in the SW 41 bucket rotor at 35,000 rpm for 16 hours at 4° C (b). *c* Nuclear-associated cytoplasm (A) and nuclear extract (B) were centrifuged in preformed CsCl gradients containing 4 per cent formaldehyde as in "b". *d* Nuclear-associated cytoplasm (A) and nuclear extract (B) from rimantadine-treated cells were centrifuged in CsCl gradients as in "b". The cells were pretreated with 50 μg/ml of rimantadine hydrochloride for one hour, and the same rimantadine-containing medium was added after infection. The gradients were fractionated and processed for acid-insoluble radioactivity


a
80S
^{14}C (cpm × 10^{-3})
8
4
5 10 15 20
b
1.29
^{14}C (cpm × 10^{-2})
4
2
1.40 1.33 1.26
c
1.34
1.29
^{14}C (cpm × 10^{-2})
4
2
1.37 1.29 1.21
d
1.29
^{14}C (cpm × 10^{-3})
2
1
1.44 1.36 1.28 1.20
♀
A

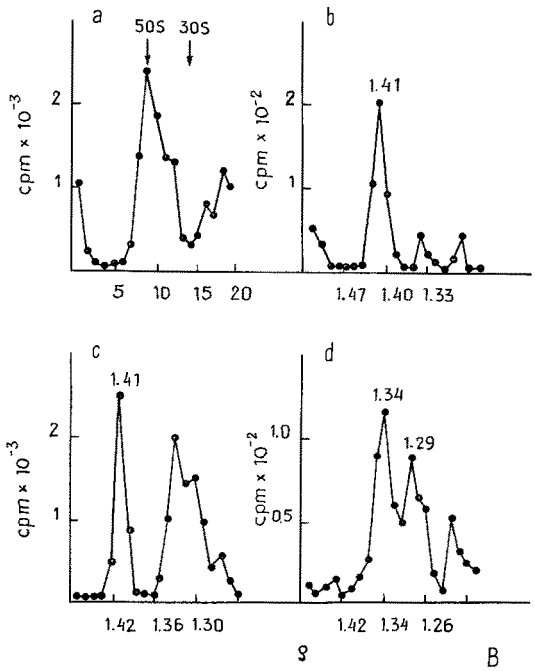

a
50S 30S
cpm × 10^{-3}
2
1
5 10 15 20
b
1.41
cpm × 10^{-2}
2
1
1.47 1.40 1.33
c
1.41
cpm × 10^{-3}
2
1
1.42 1.36 1.30
d
1.34
1.29
cpm × 10^{-2}
1.0
0.5
1.42 1.34 1.26
♀
B

Fig. 1

Table 1 shows the distribution of parental virus radioactivity in untreated and rimantadine treated cells. The amount of cell-associated input virus radioactivity was nearly the same in both cases. The radioactivity in the cytoplasmic fraction, which was mainly due to the virus adsorbed on cellular membranes (1) was also similar in untreated and drug-treated cells. This demonstrates that rimantadine does not interfere with the process of virus adsorption and penetration. On the other hand, rimantadine drastically changed the distribution of input virus radioactivity between nuclear-associated cytoplasm and nuclei: the radioactivity in nuclear-associated cytoplasm was move than twice that of drug-treated cells while in the nuclei it was reduced by at least a factor of two. Most of the intranuclear radioactivity could be extracted from rimantadine-treated cells by 0.16 M NaCl.

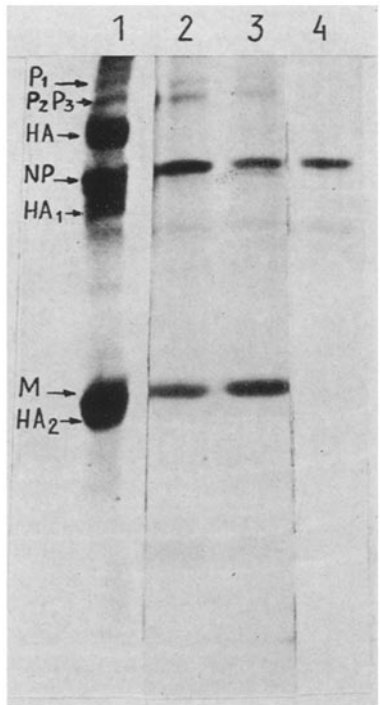


Fig. 2. Polypeptides of intracellular input virus structures. MDCK cells were fractionated 30 minutes after infection with WSN virus prelabeled with ^{14}C-amino acids. Nuclear-associated cytoplasm from untreated *(2)* and rimantadine-treated *(3)* cells was centrifuged in CsCl gradients without formaldehyde, and polypeptides of the 1.29 structure were analysed. *4* polypeptides of the nuclear extract obtained from untreated cells. *1* polypeptides of WSN virus labeled with ^{14}C-amino acids were taken as markers. The polypeptides were analysed by electrophoresis in 10 per cent polyacrylamide gels according to Laemmli (4)

The sedimentation and buoyant density analysis of parental virus structures in subcellular fractions is shown in Fig. 1. It is seen that virus structures in nuclear-associated cytoplasm sediment more rapidly in glycerol gradients than parental virus RNPs in nuclear extracts [Figs. 1A (a) and 1B (a)]. When recentrifuged in

CsCl, these structures band at 1.29 g/ml with a shoulder at 1.25 g/ml [Fig. 1 A (b)]. The total material from untreated cells banded in CsCl heterogeneously, while the same material from rimantadine-treated cells banded more homogeneously at 1.29 g/ml [Fig. 1 A (c—d)]. When the proteins of the 1.29 structure were analysed by PAGE nucleocapsid proteins P and NP and matrix protein M were revealed (Fig. 2).

These results are compatible with the suggestion that the 1.29 structure represents virus cores that are formed from input virus particles and accumulate in nucleus-associated cytoplasm. In accordance, the electron microscope examination of the 1.29 component from nucleus-associated cytoplasm revealed the presence of round particles containing helical strands (Fig. 3).

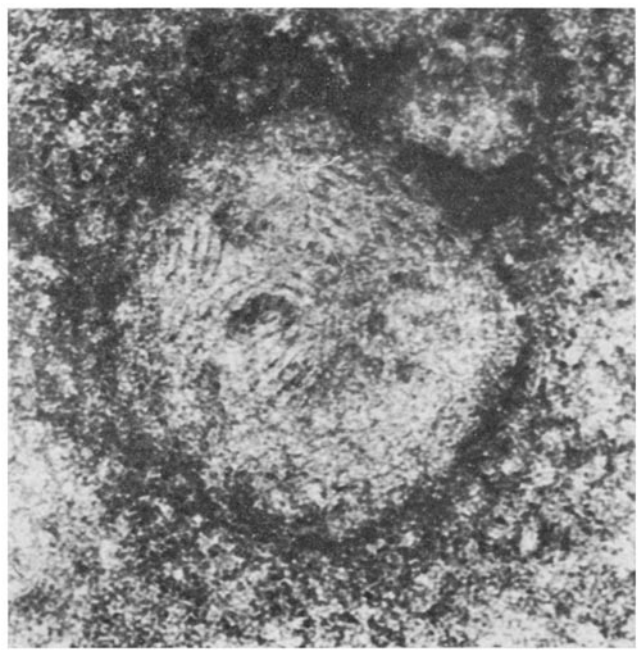


Fig. 3. Electron micrograph of the 1.29 material obtained from nuclear-associated cytoplasm. MDCK cells were fractionated and nuclear-associated cytoplasm was analysed as in Fig. 1 Ac, and the 1.29 structures were investigated for electron microscopy after negative staining with uranyl acetate ($\times$ 380,000)

The parental structures extracted from the nuclei of untreated cells by 0.16 M NaCl were previously identified as virus RNPs: they sedimented at 40—70 S in glycerol gradients, banded at 1.41 g/ml in CsCl gradients, and PAGE analysis of their proteins revealed nucleocapsid protein NP and no M protein (1, see also Figs. 1 B and 2). Low amounts of RNPs with a buoyant density of 1.34 g/ml were isolated as well from the nuclei of rimantadine-treated cells [Fig. 1 B (d)].

The autoradiographs of cells 30 minutes after infection with ^{3}H-uridine virus are shown in Fig. 4. A striking difference in grain distribution was seen in rimant-

adine-treated cells as compared to untreated cells. Most of the grains in untreated cells were located above the nuclei, while in rimantadine-treated cells the nuclei were practically free of grains, and the grains accumulated in the cytoplasm near the nucleus. This region of the cytoplasm differed from the residual part in poor staining suggesting that it contained some specific cellular organelles. The enlargement of this region in some rimantadine-treated cells could mean that these organelles were specifically affected by the drug.

The results presented allow to suggest that in infected cells cores are formed from input virus particles representing apparently intermediates in virus un-

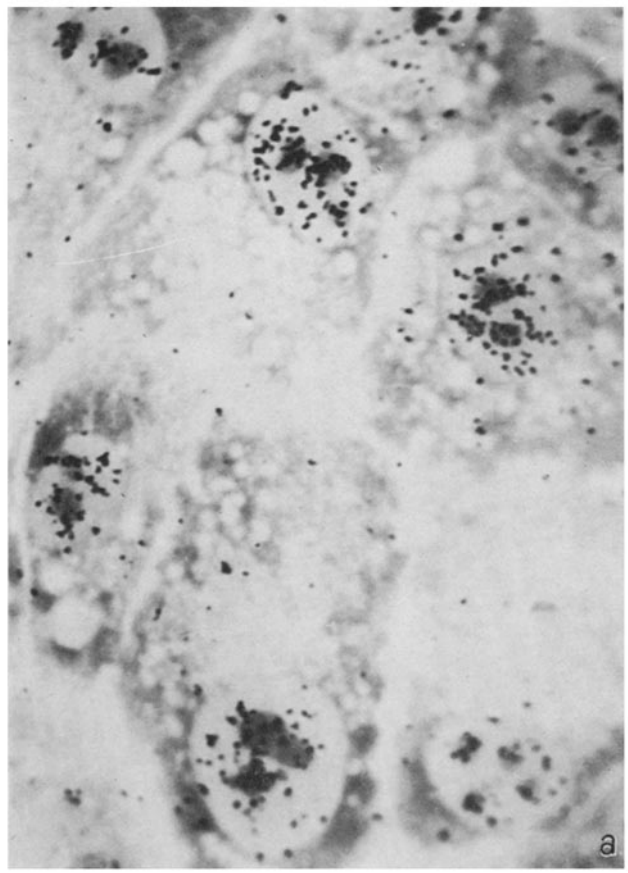


Fig. 4a

Fig. 4. Autoradiographs of MDCK cells 30 minutes after infection with WSN virus labeled with ^{3}H-uridine. Cells grown on cover slips in Leiton tubes were infected with ^{3}H-uridine labeled virus (sp.act. about 10^4 cpm/HA unit or 1—4 cpm/PFU), after 30 minutes incubation at 37° C they were washed, fixed, dipped in photographic emulsion and stored at +4° C for autoradiographic exposure. After 2 weeks the specimens were developed and stained. *a* untreated cells, *b* rimantadine-treated cells. The cells in "b" were pretreated with 50 μg/ml of rimantadine hydrochloride for one hour, and the same rimantadine-containing medium was added after infection

coating. They are located in the nuclear-associated cytoplasm, while RNPs are found mainly within the nuclei. So far, the process of virus uncoating could be divided into two steps: In the first step cores are formed, which accumulate in the nuclear-associated cytoplasm. In the second step ribonucleoproteins penetrate into the nuclei. Rimantadine seems to block the second step of uncoating "freezing" the cores and preventing the liberation of the M protein. Thus, RNPs could not be released from M protein and transported into nuclei, and the nuclear stage of replication is switched off.

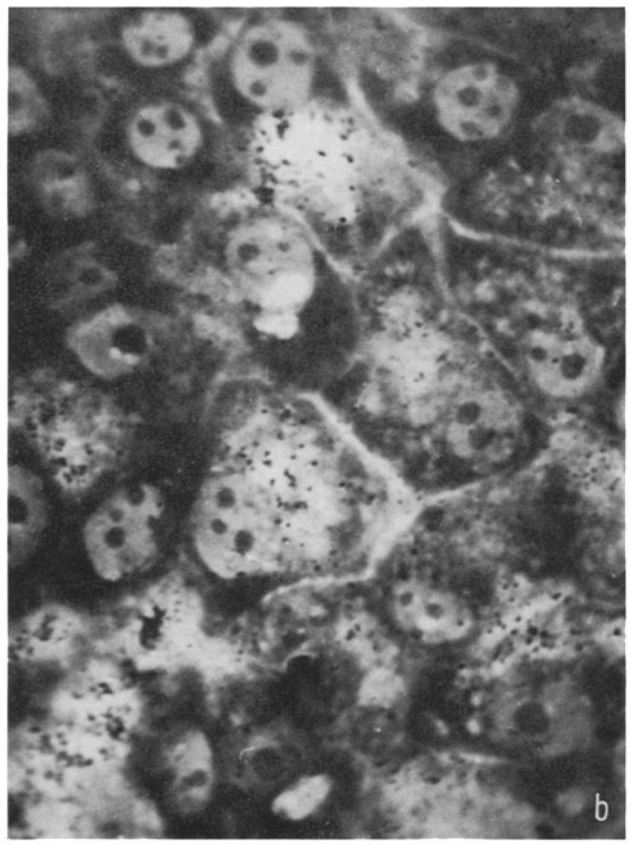


Fig. 4b

The mechanism of core "freezing" by rimantadine is unknown. Rimantadine may interact with M protein blocking further uncoating of cores, or alter the cell organells, located most probably in the nuclear-associated cytoplasm so that they could not support the second step of virus uncoating.

The mode of action of rimantadine on uncoating described here is in good agreement with data indicating a dependence of sensitivity to rimantadine on the gene for the M protein (2, 5).

References

1. BUKRINSKAYA, A. G., VORKUNOVA, G. K., VORKUNOVA, N. K.: Cytoplasmic and nuclear input virus RNPs in influenza virus infected cells. J. gen. Virol. **45**, 557—567 (1979).
2. HAY, A. J., KENNEDY, N. C. T., SKEHEL, J. J., APPLEYARD, G.: The matrix protein gene determines amantadine-sensitivity of influenza viruses. J. gen. Virol. **42**, 189—192 (1979).
3. KOFF, W. C., KNIGHT, V.: Inhibition of influenza virus uncoating by rimantadine hydrochloride. J. Virol. **31**, 261—263 (1979).
4. LAEMMLI, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680—685 (1970).
5. LUBECK, M. D., SCHULMAN, J. L., PALESE, P.: Susceptibility of influenza A viruses to amantadine is influenced by the gene coding for M protein. J. Virol. **28**, 710—716 (1978).
6. SKEHEL, J. J., HAY, A. J., ARMSTRONG, J. A.: On the mechanism of inhibition of influenza virus replication by amantadine hydrochloride. J. gen. Virology **38**, 97 to 110 (1977).

Authors' address: Dr. A. G. BUKRINSKAYA, D.I. Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences, 16 Gamaleya str., Moscow 123098, U.S.S.R.

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