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DEPROTEINIZATION OF INFLUENZA VIRUS IN THE PRESENCE OF RIMANTADINE

V. I. BUBOVICH, G. M. RYAZANTSEVA, M. K. INDULEN

The A. M. Kirchenstein Institute of Microbiology, Academy of Sciences of the Latvian Soviet Socialist Republic, 226067 Riga, U.S.S.R.

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Summary. — Virion deproteinization and viral RNA transport to the isolated cell nuclei have been studied in the presence of rimantadine with rimantadine-sensitive influenza viruses fowl plague (H7N7), A/Krasnodar/101/59 (H2N2) and rimantadine-resistant influenza strains (WSN/H1N1 and A/Krasnodar/101/59-R). Rimantadine failed to affect deproteinization during incubation with the isolated cellular plasma membranes as well as the transport to isolated cell nuclei of the viral RNA of either sensitive or resistant strains of influenza virus. Using photosensitive viruses (labelled with neutral red) rimantadine exerted dissimilar effects on deproteinization of the sensitive and resistant influenza virus strains. The possible effects of rimantadine on influenza virus deproteinization is discussed.

Key words: influenza virus; photosensitive virus; viral particles, deproteinization; rimantadine

Introduction

The mechanism of action of adamantane derivatives with anti-influenza activity — rimantadine and amantadine — is not quite clear. At present, most investigators believe that they inhibit either the stage of deproteinization of viral particles (Koff and Knight, 1979; Bukrinskaya *et al.*, 1980), or the early stage of primary transcription (Oxford and Petterson, 1980; Indulen and Kalninya, 1980; Golubev *et al.*, 1982).

We have followed in the presence of rimantadine the deproteinization of rimantadine-sensitive and rimantadine-resistant influenza virus strains, and also the transport of parental genome to the cellular nuclei.

Materials and Methods

Cells. Primary trypsinized chick embryo fibroblasts (CEF), continuous culture of dog kidney cells (MDCK), chorioallantoic membrane cultures (CAM).

Viruses. Fowl plague virus (FPV), strain "Weibridge" (H7N7), human influenza viruses: WSN (H1N1), A/Krasnodar/101/59 (H2N2) and rimantadine-resistant A/Krasnodar-R (prepared by R. L. Feldblum); trypsin was used (1 μg/ml) for treatment of human influenza viruses cultured in MDCK cells.

Photosensitive viruses (P-virus) were prepared by passages in tissue culture in the presence of neutral red (10 μg/ml) as described by Kato and Eggers (1969). The experiments with P-viruses were carried out in red light.

RNA-labelled virus was grown in tissue culture in the presence of ^{3}H-uridine (1.0—2.0 MBq/ml). The virus was concentrated and purified using the technique of Pons and Hirst (1968), except for purification in sucrose density gradient.

Photoinactivation of P-virus and the count of infective centers were carried out as described by Kato and Eggers (1969).

Cellular plasma membranes were isolated using the tris-method of Warren *et al.* (1966).

Estimation of "uncoating" activity of cellular plasma membranes. One sample contained 0.2 to 0.5 mg of membrane protein. To the membrane pellet suspended in 0.2 ml of Tris-HCl buffer (0.05 mol/l Tris, pH 7.4; 0.05 mol/l $MgCl^2$) 0.2 ml of RNA-labelled virus was added (2×10^5 HA units per sample). The adsorption lasted for 2 hr at 4 °C. Nonadsorbed virus was three times washed off with the same buffer. The virus-membrane complex was suspended in a given vol of the same buffer. To membrane aliquots ATP (3 μmol/l) alone, rimantadine alone or both were added. The incubation lasted for 1 hr at 36 °C, the control sample was incubated at 4 °C. Radioactivity was counted in the trichloracetic acid (TCA) insoluble fraction.

Isolation and purification of nuclei. The infected cells were washed by cool PBS and mechanically removed. The nuclei were isolated and purified as described by Bukrinskaya *et al.* (1980). Equal vol of 10% TCA were added to the citric acid and purified nuclei fraction. TCA-insoluble fraction was dissolved in 1 ml of SOLUENE-100 (tissue solvent) and mixed with 10 ml of scintillation fluid consisting of 5.5 g of PERMABLEND III in 1 l of toluene.

Results

Rimantadine-sensitivity of influenza viruses

Rimantadine-sensitivity of the tested influenza virus strains was studied in CEF (FPV and WSN) and MDCK (WSN and A/Krasnodar) cultures under agar overlay and in CAM. Before infection the cells were incubated for 30 min at 37 °C in the presence of rimantadine; after infection it was added into the

Table 1. Deproteinization of RNA-labelled influenza viruses during incubation with isolated cellular plasma membranes in the presence of rimantadine

Experimental system	Temperature of incubation (°C)	ATP (3 μmol/l)	TCA-insoluble RNA fraction (c.p.m.)			„Uncoating"***		
			FPV*	WSN**	A/Krasnodar**	FPV	WSN	A/Krasnodar
Membranes	(control)	—	5212	1048	1006	0	0	0
+	4							
virus	36	—	6547	1246	1260	0	0	0
		+	3048	574	271	41.5	45.2	73.1
The same		—	5200	1059	994	0	0	0
+	36							
remantadine (50 μg/ml)		+	2740	618	182	47.5	41.0	81.0

Notice: *The virus was grown in and membranes were isolated from CEF cells;
**The virus was grown in and membranes were isolated from MDCK cells;
***Deproteinization as characterized by acid-soluble radioactivity counts, related to controls (100%).

Table 2. Deproteinization of intracellular influenza virus in the presence of rimantadine (the study of the loss of the virus photosensitivity)

Experimental system	Photo-inacti-vation (15 min)	Number of infective centres (PFU/ml)							
		FPV*		WSN**		A/Krasnodar**		A/Krasnodar-R**	
		0 hr	3 hr	0 hr	3 hr	0 hr	3 hr	0 hr	3 hr
Cells + virus (control)	−	1.1×10^5	6.6×10^4	2.3×10^3	8.1×10^3	1.2×10^3	3.5×10^2	2.0×10^2	1.3×10^2
	+	1.8×10^3	2.2×10^4	1.1×10	2.8×10^3	0.5×10	0.2×10^2	0	0.1×10^2
Light-resistance (%)		1.6	32.5	0.5	35.0	0.4	7.2	0	7.7
The same + rimantadine (25 μg/ml)	−	8.0×10^4	9.4×10^4	3.2×10^3	2.4×10^3	7.0×10^2	3.4×10^2	2.9×10^2	1.8×10^2
	+	7.5×10^2	1.2×10^3	0.7×10^2	1.1×10^3	0	0	0	2.8×10
Light-resistance (%)		0.9	1.2	2.2	45.8	0	0	0	14.9

Notice: Cells were infected at multiplicity of 0.01—0.5 PFU per 1 cell or at 0.01—0.5 EID_{50} per 1 cell. Adsorption was carried out for 40 min at 4°C. Then the cells were twice washed with cold medium No. 199 and incubated for 3 hr at 37°C. At 0 hr and 3 hr photoresistant infective centres were determined. Rimantadine was added 30 min prior to virus inoculation and then to the medium for 3hr. The experiments (not less than 3 with each virus) were carried out in CEF* and in MDCK** cells.

Table 3. FPV deproteinization in CEF cells in the presence of rimantadine[1]) and other inhibitors

Experimental system	Photoinactivation (15 min)	Number of infective centers 3 hr post-infection (PFU/ml)		
		no inhibitors	CHM (10 μg/ml)	AzU (2.0 mg/ml)
Cells + virus (control)	—	3.8×10^5	7.4×10^5	1.2×10^5
	+	1.8×10^5	4.4×10^5	0.9×10^5
Light-resistance %		47.5	60.0	75.0
The same + rimantadine (25 μg/ml)	—	0.2×10^5	2.3×10^5	0.8×10^5
	+	0.7×10^3	7.1×10^3	5.8×10^3
Light resistance (%)		3.4	3.1	6.9

[1]) Experimental design as in Table 2.

agar overlay. In experiments on CAM rimantadine was given together with the virus. The results indicated that rimantadine at concentration of 25 μg/ml entirely inhibited FPV and A/Krasnodar reproduction at input multiplicity of 100 PFU per monolayer or 100 EID_{50} per 1 CAM sample, respectively. Under analogous conditions the strains WSN and A/Krasnodar-R appeared rimantadine-resistant.

Virus deproteinization in vitro in the presence of rimantadine

Deproteinization was studied using viruses labelled with ^{3}H-uridine, incubated with plasma membranes isolated from CEF or MDCK cells. As shown by Križanová *et al.* (1971), the influenza virus is disintegrated in the presence of ATP under these conditions. It follows from the results presented in Table 1, that viral RNA in the survival structures produced during uncoating is equally sensitive to RNases contained in the plasma membranes, both in the presence and in the absence of rimantadine using either sensitive or resistant strains.

Virus deproteinization in cells in the presence of rimantadine

In these experiments photosensitive variants of the virus have been used. Earlier, Kato and Eggers (1969) have shown that the loss in P-virus sensitivity to visible light soon after infection is related to the stage of deproteinization of viral particle. We have studied the effect of rimantadine on the production of light-resistant infective centres using sensitive and resistant strains. The results of typical experiments are presented in Table 2. At the end of adsorbtion (0 hr) all virus strains were highly sensitive to the visible light and almost entirely inactivated (97.8—100 %). After 3 hr incubation at 37 °C, 30—40% of FPV and WSN on average was converted to the light-resistant form. The intensity of conversion A/Krasnodar and A/Krasnodar-R viruses

Table 4. Transport of influenza virion RNA to the isolated cell nuclei in the presence of rimantadine

Virus	Rimantadine (50 μg/ml)	Radioactivity (%)	
		citric acid fraction	nuclei
A/Krasnodar	−	23.3±13.9	76.7±13.9
	+	25.8±14.0	74.2±14.0
A/Krasnodar-R	−	20.0± 5.1	80.0± 4.1
	+	20.8± 0.4	79.2± 0.4
WSN	−	66.4±12.4	33.6±12.4
	+	64.1±12.3	35.9±12.3

Notice: MDCK cells were infected at the multiplicity of 5—10 EID_{50} per cell and RNA-labelled with ^{3}H-uridine. The adsorption was carried out for 1 hr at 4°C. Then the cells were washed in medium No. 199, quickly heated up to 37°C, incubated for 30 min at 37 °C and the nuclei were isolated. Rimantadine was added 1 hr prior to infection to the inoculum and to the medium. The labelled virus was prepared in MDCK cells in the presence of trypsin (1 μg/ml). The results were treated statistically ($P = 0.05$).

was somewhat lower (6.4±0.9 and 11.8±5.9%, respectively). In the presence of rimantadine (25 μg/ml) all FPV and A/Krasnodar virus remain photosensitive at 3 hr postinfection ($p \leq 0.05$), unlike WSN and A/Krasnodar-R in which the photosensitivity is significantly reduced ($p > 0.05$). Thus, in sensitive strains rimantadine blocks the conversion of P-virus to the visible light-resistant form not affecting the resistant viruses.

To study the loss of virus photosensitivity, we used cycloheximide (CHM) simultaneously inhibiting the virus and host protein synthesis as well as 6-azauridine (AzU) inhibiting the virus and host mRNA syntheses. CHM was added 30 min prior to the P-virus, while AzU was given 2 hr prior to infection and then to the culture fluid. By 24 hr before infection, the growth medium has been replaced with Hank's solution to achieve a depletion of the pyrimidine pool. As seen in Table 3, after simultaneous addition of CHM and rimantadine or AzU and rimantadine to infected cells the virus remained almost entirely sensitive to light. These data indicate that rimantadine exerts its inhibitory action before transcription and/or replication of viral genome, and the loss of photosensitivity appears to coincide with virion deproteinization.

Transport of virion RNA to the isolated cell nuclei in the presence of rimantadine

In these experiments we estimated the amount of ^{3}H-labelled virion RNA in purified nuclei isolated from rimantadine treated (50 μg/ml) and untreated infected cells. Table 4 shows the distribution of the label between the citric acid fraction and the nuclei. In the presence of rimantadine, the amount of ^{3}H-RNA found in the cell nuclei was the same as in control sample without rimantadine. Thus, rimantadine did not prevent influenza virus RNA transport to isolated cell nuclei.

Discussion

It has been reported that due to rimantadine-block of the second stage of influenza virus deproteinization, parent subviral structures cannot get into the nuclei, but are accumulated at the nuclear membrane (Bukrinskaya *et al.*, 1980). Our data are not consistent with this. Rimantadine fails to block the transport of parent RNA of both sensitive (A/Krasnodar) and resistant strains (A/Krasnodar-R and WSN) of the influenza virus to the isolated cell nuclei. Similar results were obtained by Golubev *et al.* (1982). Nevertheless, our data on rimantadine-inhibition of the loss of the sensitivity to the visible light exposure in the photosensitive influenza virus suggest that rimantadine inhibits either the second stage of deproteinization itself or yet unknown phenomenon responsible for the very beginning of primary transcription. It has been shown that some strains binding with the nucleic acid of RNA and DNA viruses, can inhibit viral reproduction (Melnick and Wallis, 1975). On the other hand, it has been established that in enteroviruses the loss of photosensitivity is related to the stage of deproteinization (Eggers, 1977). We have shown that the loss of photosensitivity of influenza virus is not related to mRNA or protein synthesis de novo and is likely to reflect the process of virion deproteinization (see Table 3). Thus, the inhibitory effect of rimantadine occurred before viral mRNA synthesis. However, it cannot be ruled out that rimantadine affects the function of a cellular factor needed for deproteinization.

Križanová *et al.* (1971) demonstrated that plasma membranes isolated from CEF in the presence of exogenous ATP disintegrate the native influenza virus (WSN). Our data indicate that the interaction of ^{3}H-uridine-labelled influenza viruses with isolated plasma cellular membrane results in the release of the structures containing RNase-sensitive RNA; these structures seem to be of RNP type, since the RNA contained in them is RNA-resistant, unlike the RNA contained in the nucleoids (Zvonarev *et al.*, 1980). Rimantadine does not prevent the interaction of viral and cellular membranes in vitro (see Table 1), nor does it affect the process of virus deproteinization under these conditions.

On the basis of our results we can make 2 suggestions concerning the phenomena observed in the infected cell in the presence of rimantadine. 1. If the virus looses its photosensitivity in the cytoplasm as a result of the second stage of deproteinization, then the disturbance of this stage in the presence of rimantadine might lead to the penetration of functionally inactive nucleocapsids, and not of the viral RNPs, into the nuclei. This possibility can not be ruled out, since parent protein M of the influenza virus was detected in the purified nuclei of infected cells (Hudson *et al.*, 1978). 2. If the loss of virus photosensitivity takes place in the nucleus resulting from the so-called conformational deproteinization, when it changes its permeability without loosing the protein subunits (pretranscriptional state of RNP — Dimmock, 1982), so then rimantadine can prevent the conformational changes of influenza virus RNP.

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