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Influence of Membrane (M) Protein on Influenza A Virus Virion Transcriptase Activity In Vitro and Its Susceptibility to Rimantadine

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The transcriptase activity of influenza A virus ribonucleoproteins was inhibited by 42 to 49% in vitro in the presence of membrane (M) protein. The addition of M protein to the system of ribonucleoprotein preparations isolated from rimantadine-sensitive or rimantadine-resistant influenza virus strains, as well as the addition of M protein isolated from a sensitive strain, in the presence of rimantadine further inhibited the transcriptase activity of such complexes by approximately 40%. In the system containing the same ribonucleoprotein preparations, but with M protein isolated from a rimantadine-resistant influenza virus strain, the transcriptase activity was not sensitive to rimantadine. The data show that M protein can influence the activity of influenza A virus virion transcriptase and that the susceptibility of influenza virus to rimantadine may be due to the peculiarities of M protein.

Membrane (M) protein, which constitutes about 40% of the total amount of virion proteins, forms the internal membrane of a virion. The exact function of M protein in the reproduction of orthomyxoviruses is not yet clear, although results of studies on temperature-sensitive mutants confirm that this protein participates in the late stages of virion morphogenesis (14).

Carroll and Wagner (3) recently showed for a vesicular stomatitis virus model that when M protein isolated from virions was added to a system containing vesicular stomatitis virus ribonucleoprotein (RNP), the transcriptase activity was decreased by approximately 40%. On the other hand, it was found out that the susceptibility or resistance of orthomyxoviruses to amantadine and its derivatives was due to a mutation of a gene coding for M protein (6, 12). Also, there are data showing that amantadine and its derivatives inhibit the reproduction of orthomyxoviruses at early stages, without apparently impairing the process of adsorption and penetration of virions into a cell (4, 7, 9, 15).

In this paper, we investigated the probable influence of M protein on the transcriptase activity of influenza virus RNP in vitro and the role of this protein in the manifestation of susceptibility or resistance of this virus to rimantadine.

MATERIALS AND METHODS

Viruses. The following influenza viruses were used: A/PR/1/74 (H3N2), sensitive to rimantadine (reproduction in chicken embryos in the presence of 2 mg of rimantadine was decreased by 2 $\log_{10}$ 50% egg infective doses); A/Victoria/72/R (H3N2), resistant to rimantadine and obtained by multiple passages in chicken embryos in the presence of rimantadine (reproduction in chicken embryos of a rimantadine-sensitive parent strain in the presence of 2 mg of rimantadine was decreased by 1.5 to 2 $\log_{10}$ 50% egg infective doses, but reproduction of a rimantadine-resistant variant under the same conditions was not inhibited at all). The viruses were grown in the allantoic cavity of 11-day-old embryonated eggs.

Evaluation of transcriptase activity in experiments in vitro. In the majority of experiments, we used the method described by Bishop et al. (1) and modified by Blagoveshchenskaya and Ghendon (2). The system contained the following in a 125-μl volume: 8 μM Tris-hydrochloride, pH 8.2; 1 μM $MgCl_2$; 13 μM NaCl; 0.5 μM β-mercaptoethanol; 0.12 μM $MnCl_2$; 0.25% Nonidet P-40; 0.2 μM ATP; 0.1 μM GTP; 0.1 μM UTP; and 2 μCi of [^{3}H]UTP (Amersham Corp.; specific activity, 11.7 Ci/mmol). In a number of experiments, 0.5 μM GpG was added to the system as well, since this preparation stimulates the activity of a virion transcriptase of orthomyxoviruses in vitro (13).

Virions, RNP, and M protein were added to the system in samples of 70 to 100, 50, and 80 μg, respectively, in a 0.25% Nonidet P-40 solution; in this instance, the amount of Nonidet P-40 in the system was also 0.25%. The system was incubated at 33.5°C for 1 h. The protein concentration was determined as described by Lowry et al. (11).

Purification of viruses. The purification of viruses was performed in potassium tartrate as described by Kingsbury (10).

RNP isolation. Purified virions were treated with a mixture containing 1% Nonidet P-40, 0.1% deoxycholate, 0.2 M urea, and 0.01 M β-mercaptoethanol at 22°C for 15 min. The material was then layered onto the top of a preformed linear 15 to 30% glycerol gradient in ST buffer (0.01 M Tris-hydrochloride, pH

7.4; 0.15 M NaCl) and centrifuged (Spinco L-5-65 centrifuge and SW27 rotor) at 18,000 rpm and 4°C for 16 h. RNP-containing gradient fractions (determined by the optical density at 260 nm) were pooled, and RNP was sedimented by centrifugation (Spinco L-5-65 centrifuge and rotor Ti 65 at 50,000 rpm and 4°C for 90 min). The pellets were resuspended in ST buffer and dialyzed against ST buffer to a 1-ml volume on XM-300 wide-pore filters of an Amicon system. Polyacrylamide gel electrophoresis analysis showed that by this method RNP contained P1, P2, and P3 proteins and nucleoprotein (NP) and was free of M protein and those of the virion external membrane (data not shown).

M protein. M protein was isolated from purified virions by the method of Gregoriades (5) and suspended in 0.25% Nonidet P-40. Polyacrylamide gel electrophoresis revealed only M protein free from any other virion proteins in the isolated preparations (data not shown).

Rimantadine. The preparation of rimantadine synthesized at the Institute of Organic Synthesis, Academy of Science, Latvian SSR, Riga, was used.

RESULTS

Influence of M protein on transcriptase activity of influenza virus RNP. In the first series of experiments, we studied the influence of M protein added to the system containing influenza virus RNP. In determining the amount of M protein added to the system, we proceeded from the fact that, according to data from the polyacrylamide gel electrophoresis analysis, in virions of the influenza virus strain that was investigated, the NP/M polypeptide ratio was 1:1.6 (data not shown). Since the main portion of proteins in RNP was NP, we added 50 μg of RNP and 80 μg of M protein to the system.

Table 1 shows that in all five experiments the addition of M protein to the system decreased the transcriptase activity of virion RNP, with the degree of inhibition being 42 to 49%.

Influence of rimantadine on transcriptase activity of influenza virus virions in vitro. Having obtained the above-mentioned results regarding the influence of M protein on the transcriptase activity of influenza virus virions and considering the data available in the literature regarding a possible role of M protein in the manifestation of susceptibility or resistance of orthomyxoviruses to amantadine and its derivatives (6, 12), we decided to study the influence of M protein on the activity of influenza virus virion transcriptase in the presence of rimantadine.

In the literature there are contradictory data regarding the influence of amantadine and its derivatives on the function of orthomyxovirus virion transcriptase in vitro (8, 15; J. S. Oxford, S. Patterson, and R. Dourmashkin, Abstr. Int. Virol. Congr. IV, p. 128, 1978), which might be due to the peculiarities of proteins of different orthomyxovirus strains or to the differences in the composition of the systems used for the evaluation of transcriptase activity.

Using different rimantadine concentrations, we found (Table 2) that in the system containing purified virions of A/PR/1/74 influenza virus, considerable inhibition of transcriptase activity (by 44 to 57%) was observed only in the presence of 200 μg of rimantadine per 0.125 ml of the system. At lower concentrations of rimantadine, inhibition of transcriptase activity was poorly expressed. It should be noted that in the presence of 200 μg of rimantadine, a similar level of inhibition of transcriptase activity (about 50%) was observed in both the system containing that and not containing GpG (data not shown). The

TABLE 1. *Effect of M protein on transcriptase activity of influenza A virus RNP in vitro*

Examined material	Incorporation of [^{3}H]UMP (cpm/mg of protein)			
	Expt 1	Expt 2	Expt 3	Expt 4
RNP	32,720	28,560	38,210	24,900
RNP plus M protein	18,800	16,310	21,010	12,420

TABLE 2. *Effect of rimantadine on the activity of influenza A virus virion transcriptase in vitro*

Examined material	Expt	Rimantadine concn (μg/0.125 ml)	Incorporation of [^{3}H]UMP	
			cpm/mg of protein	Inhibition (%)
A/PR/1/74 virions (rimantadine sensitive)	1	0	12,080	10
		50	10,860	19
		100	9,790	51
		200	5,990	
	2	0	16,910	
		100	14,040	17
		200	7,270	57
	3	0	11,300	
		200	6,330	44
	4	0	8,310	
		200	3,990	52
A/Victoria/72/R virions (rimantadine resistant)	1	0	15,910	
		200	14,800	2
	2	0	17,270	
		200	19,030	Absent
A/PR/1/74 RNP	1	0	26,920	
		200	27,820	Absent
	2	0	23,250	
		200	20,930	10
	3	0	20,610	
		200	22,720	Absent
	4	0	32,460	
		200	35,640	Absent

inhibiting effect of a rimantadine concentration of 200 μg/0.125 ml was specific, since in parallel experiments with a rimantadine-resistant A/Victoria/73/R strain of influenza virus, inhibition of transcriptase activity was hardly observed (Table 2). Besides, as shown in Table 2, the transcriptase activity of A/PR/1/74 influenza virus RNP was not inhibited at all in the presence of 200 μg of rimantadine.

Influence of M protein on the activity of influenza virus virion transcriptase in the presence of rimantadine. Bearing in mind that the transcriptase activity of RNP (i.e., the preparation that did not contain external membrane proteins and M protein) isolated from a rimantadine-sensitive influenza virus strain was not sensitive to rimantadine, unlike that of complete virions, we decided to study to what degree the addition of M protein to the influenza virus RNP-containing system would influence the susceptibility of the transcriptase activity to rimantadine.

Table 3 shows that the addition of M protein to the RNP-containing system decreased the transcriptase activity by 40%, which correlates with the data of Table 1. When rimantadine (200 μg/0.125 ml) was added along with M protein to the system, the transcriptase activity was decreased further by 30 to 40%.

We then carried out experiments on the isolation of RNP and a membrane protein from the virions of a rimantadine-sensitive strain of influenza virus (A/PR/1/74) and a strain resistant to this inhibitor (A/Victoria/72/R), with the addition of RNP of a sensitive strain and M protein of a resistant strain, and vice versa, to the RNP-containing system for evaluation of transcriptase activity. The studies of the susceptibility of the transcriptase activity of such a system to rimantadine revealed (Table 4) that in the process of the complex formation of A/PR/1/74 influenza virus RNP with M protein of the same strain, the transcriptase activity, as in the previous experiments, was decreased by 40%, and the complex obtained, unlike the RNP preparation, appeared to be sensitive to rimantadine (34%

TABLE 3. *Effect of M protein on the susceptibility to rimantadine of the transcriptase activity of A/PR/1/74 influenza virus RNP (rimantadin sensitive)*

Examined material	Incorporation of [^{3}H]UMP (cpm/mg of protein)							
	Expt 1				Expt 2			
	Without rimantadine		200 μg of rimantadine		Without rimantadine		200 μg of rimantadine	
	cpm/mg of protein	Inhibition (%)	cpm/mg of protein	Inhibition (%)	cpm/mg of protein	Inhibition (%)	cpm/mg of protein	Inhibition (%)
RNP	22,660		24,820	Absent	30,250		28,810	5
RNP plus M protein[a]	13,900	40[b]	8,710	40[c]	18,810	40[b]	13,420	29[c]

[a] In the calculation of [^{3}H]UMP per milligram of protein, only RNP was considered.
[b] Inhibition in regard to RNP preparation.
[c] Inhibition in regard to RNP plus M protein preparation without rimantadine.

TABLE 4. *Susceptibility to rimantadine in vitro of the transcriptase activity of a rimantadine sensitive influenza virus RNP complex with M protein of a resistant strain, and vice versa*

Examined material	System			
	Without rimantadine		200 μg of rimantadine	
	Incorporation of [^{3}H]UMP (cpm/mg of protein)	Inhibition (%) in regard to RNP activity	Incorporation of [^{3}H]UMP (cpm/mg of protein)	Inhibition (%) in the presence of rimantadine
PR/1 RNP	40,200		40,400	Absent
PR/1 RNP plus PR/1 M[a]	25,550	40	16,750	34[b]
PR/1 RNP plus Victoria M[a]	30,806	24	27,450	11[b]
Victoria RNP	24,420		25,140	Absent
Victoria RNP plus Victoria M[a]	17,260	28	16,210	7[b]
Victoria RNP plus PR/1 M[a]	13,840	41	7,650	44[b]

[a] In the calculation of [^{3}H]UMP per milligram of protein under the conditions of the experiment, only the protein added to the system with the RNP preparation was considered.
[b] Inhibition in regard to RNP plus M protein without rimantadine.

inhibition). In the formation of a complex of A/PR/1/74 virus RNP with M protein of a resistant strain, A/Victoria/72/R, the transcriptase activity was decreased by 24%, and the complex obtained was not very sensitive to rimantadine (11% inhibition). In the formation of a complex of a resistant virus, A/Victoria/72/R, RNP with M protein of the same strain, the transcriptase activity was decreased by 28%, with the complex obtained being practically insensitive to rimantadine (7% inhibition). When M protein isolated from a sensitive virus, A/PR/1/74, was added to the system containing RNP of A/Victoria/72/R, the transcriptase activity of the complex was decreased by 41%, with the complex being highly sensitive to rimantadine (inhibition of the transcriptase activity by 44%).

DISCUSSION

The results of the experiments showed that the addition of 80 μg of M protein isolated from purified virions to the system containing 50 μg of influenza A virus RNP decreased the transcriptase activity in the experiments in vitro by 42 to 49%. In the experiments carried out by Carroll and Wagner (3), the transcriptase activity was decreased by 33 to 43% by the addition of 130 to 400 μg of M protein per ml to the system containing 80 μg of vesicular stomatitis virus RNP per ml; in the system containing 400 μg of RNP per ml and 360 μg of M protein per ml, the transcriptase activity was inhibited by 44%. It should be noted that in our experiments the addition of proteins of the virion external membrane (hemagglutinin and neuraminidase) to the system containing influenza A virus RNP did not affect the transcriptase activity (unpublished data). The results obtained testify to the fact that in large RNA-containing viruses M protein can influence the activity of virion transcriptase.

The experiments revealed that when M protein isolated from a rimantadine-sensitive influenza virus strain was added to the system containing RNP isolated from the same strain, the transcriptase activity of the complex appeared to be almost as sensitive to rimantadine as was the native virus. On the other hand, when M protein isolated from a rimantadine-resistant influenza virus strain was added to the system containing RNP of a sensitive virus, the transcriptase activity of such a complex was not inhibited by rimantadine. The transcriptase activity of RNP isolated from a rimantadine-resistant influenza virus strain in a complex with M protein of the same strain was resistant to rimantadine, whereas the transcriptase activity of the complex of the same RNP with M protein isolated from a sensitive strain was suppressed by 44%. These data make it possible to conclude that both susceptibility and resistance of influenza viruses to rimantadinol are due to M protein peculiarities. One can assume that in the presence of this inhibitor, M protein changes conformation and complicates a function of proteins contained in RNP which accomplishes the transcription or complicates the transport of nucleotides to RNP. Thus, rimantadine, inhibiting the process of influenza virus transcription, does not appear to affect virus proteins accomplishing the transcription, but impairs this process indirectly by means of M protein.

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