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ACTION OF RIMANTADINE ON THE STRUCTURE OF INFLUENZA A VIRUS HAEMAGGLUTININ

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Summary. — Rimantadine prevents the conformational changes of influenza virus haemagglutinin (HA) caused by acid pH and the acquisition of sensitivity to trypsin, protects the haemolytic activity from inactivation and prevents the morphological changes of HA spikes on the virus surface.

Key words: *influenza virus*; *haemagglutinin*; *rimantadine*

Introduction

Like other enveloped viruses, influenza virus introduces its genome into the cell by membrane fusion. Influenza virus mediated membrane fusion proceeds within a narrow pH range (5.0—5.5) in endocytic vesicles (March, 1984). One of influenza virus surface glycoproteins — the haemagglutinin (HA) — undergoes at acid pH a conformational change needed for the membrane fusion (Skehel *et al.*, 1982; Wharton *et al.*, 1986). The change in HA conformation at acid pH is evident from the appearance of sensitivity to trypsin, from the aggregation and some other properties (Skehel *et al.*, 1982). The change of HA conformation at acid pH can be observed on both the free HA (Skehel *et al.*, 1982) and on the intact virus particles (Ruigrok *et al.*, 1984, 1986). Data reported in the present paper demonstrate that rimantadine prevents conformational change of influenza A virus HA suppressing membrane fusion.

Materials and Methods

Fowl plague virus (*FPV*) strain Weybridge (H7N7) was prepared by infection of 10-day-old chick embryos. The virus was concentrated by precipitation with polyethylene glycol 6000 (Merck) and purified in sucrose density gradient according to the procedure of Skehel and Schild (1971). The surface glycoproteins were isolated by means of octyl glycoside (n-octyl-β-D-glucopyranoside; Calbiochem) solubilization according to the method of Sato *et al.* (1983). For preparation of ^{14}C-amino-acid-labelled surface glycoproteins 3.7 MBq from a mixture of ^{14}C-valine, ^{14}C-leucine, and ^{14}C-phenylalanine (UVVVR, Č.S.S.R.) was added into FPV-infected embryos.

Trypsin (*Calbiochem*) *digestion* was carried out according to the method of Skehel *et al.* (1982) at a protein to trypsin ratio of 20 : 1 (w/w) for 10 min at 20 °C, pH 7.5. The reaction was terminated by acetone precipitation. Virus polypeptides were analysed in 10 % polyacrylamide geel (PAG) under reducing conditions.

To study the action of rimantadine (α-methyl-1-adamantane methylamine hydrochloride) the drug was obtained from the Institute of Organic Synthesis, Academy of Sciences of Latvian Soviet Socialist Republic, Riga, U.S.S.R. An appropriate quantity of rimantadine was added to the isolated glycoprotein or to the whole purified virus at neutral pH.

Ribonucleoprotein (RNP) was isolated from purified virions as described (Melnikov *et al.*, 1985). Haemolytic activity of the virus was measured using chicken erythrocytes (Ghendon *et al.*, 1986). Briefly, the virus in ST buffer (0.01 mol/l tris-HCl pH 7.5; 0.1 mol/l NaCl) was incubated with chicken erythrocytes for 10 min at 0 °C, then 0.3 mol/l Na-citrate buffer, pH 5.2 was added. After incubation for 30 min at 34 °C the samples were centrifuged for 5 min at 1500 rev/min and the extinction value was measured in the supernatant at 520 nm using the spectrophotometer Beckman-35.

Results

Prevention of the inactivation of influenza virus haemolytic activity at acid pH by rimantadine

The incubation of influenza virus at acid pH in the absence of erythrocytes is known to result in irreversible inactivation of haemolytic activity (Sato *et al.*, 1983; Yewdell *et al.*, 1983). In our experiments, 3 FPV samples were used in ST buffer. To one of them (sample no. 3, Table 1) rimantadine was added at the protein to rimantadine ratio of 1 : 5. An equal volume of 0.3 mol/l citrate buffer pH 5.2 was then given to the samples Nos 2 and 3. All 3 preparations were kept at 33 °C for 20 min (test 1) or at 4 °C (test 2). Thereafter the samples from test 1 were cooled in ice-cold bath and the preparations Nos 2 and 3 from both tests were neutralized with 2 mol/l Tris-HCl pH 8.9 added in a volume of 1 : 15. All the 3 preparations were placed on the top of discontinuous glycerol gradients (70—50—20 %) prepared in ST buffer. Centrifugation in glycerol gradient was carried out in order to remove rimantadine from preparation No 3 (Table 1) and with the preparations Nos 1 and 2 to provide similar treatment conditions. After centrifugation (rotor SW-40, ultracentrifuge Beckman Model L-65) for 40 min at 30,000 rev/min, the virus-containing fractions were collected and haemolytic activity was determined as described. It can be seen from Table 1 that incubation of the virus at acid pH caused inactivation of the virus haemolytic activity (pre-

Table 1. Haemolytic activity of FPV after incubation under different conditions (OD at 520 nm; given in parenthesis is the activity expressed as a percentage of control values)

Preparation number	Virus treatment	Test 1	Test 2
1	pH 7.5 (control)	2.580 (100)	1.650 (100)
2	pH 5.2	0.320 (12)	0.132 (8)
3	Rimantadine + pH 5.2	1.611 (62)	1.430 (86)

Note. The conditions of incubation and treatment of the virus are described in the text.

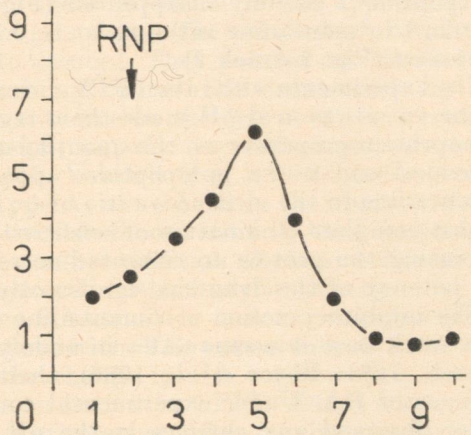

Fig. 1.
Co-centrifugation of FPV glycoproteins and RNP
^{14}C-amino-acid-labelled glycoproteins and RNP were centrifuged in 15—30 % glycerol gradient prepared on ST buffer (rotor SW-40, Beckman, Model L-65) at 27,000 rev/min for 16 hr at 4 °C). The fractions were collected, RNP position was determined by measuring the optic density at 260 nm, the position of glycoproteins was determined by ^{14}C-label.
Abscissa — fractions, ordinate — ^{14}C cpm $\times 10^{-2}$.

paration No 2). The process of inactivation was independent of the temperature (33 °C or 4 °C). If rimantadine was added to FPV preparations before lowering of pH, the haemolytic activity was "rescued" and constituted 62 to 86 % of the control (No. 1) preparation activity. It should be noted that the addition of rimantadine to control preparation followed by centrifugation through glycerol gradient was without effect on the haemolytic activity of the virus (it was as high as that of preparation No. 1). Hence, the association of rimantadine with HA was rather labile and the drug was easily eliminated by sedimentation of the virus through glycerol gradient.

Effect of rimantadine on tryptic digestion of HA

The change in HA conformation at acid pH can be easily demonstrated by the appearance of sensitivity to trypsin (Skehel *et al.*, 1982). Our experiments were carried out both with surface glycoprotein preparation and with intact virus. Surface glycoproteins were isolated from purified FPV virions as described in Materials and Methods. The resulting surface glycoprotein preparation had a sedimentation constant 28 S as demonstrated by co-centrifugation with 70 S RNP (Duesberg, and Robinson, 1967) and thus represented the so-called rosettes, i.e. aggregated glycoprotein structures (Fig. 1).

Track 5 in Fig. 2 shows that the surface glycoprotein preparation consisted of polypeptides HA0, HA1, HA2 and NA. The preparation was not sensitive to trypsin under conditions described in Materials and Methods (Fig. 2, track 1). The exposure of glycoprotein preparation to acid pH followed by neutralization and trypsin treatment led to the decrease of the quantity of HA1 polypeptide and to the appearance of new polypeptides (Fig. 2, track 4). One of them was a major protein with a molecular mass of 36,000 kD and the other was a minor protein with apparent molecular mass of 44,000 kD.

Addition of rimantadine to the glycoprotein preparation at a 1 : 1 ratio before acidification significantly decreased the quantity of polypeptide 1;

polypeptide 2 entirely disappeared (Fig. 2, track 3). When increasing the protein to rimantadine ratio up to 1 : 0.5 the effect of rimantadine was less expressed (Fig. 2, track 2).

The experiments with intact FPV virions gave similar results. The exposure of the virions to acid pH made them trypsin-sensitive. Alike to the purified glycoprotein preparations, the quantity of HA1 polypeptide was significantly decreased and 2 new polypeptides appeared (Fig. 2, track 8). Addition of rimantadine to the virus in a ratio of 1 : 1 followed by acidification led to an almost complete elimination of sensitivity to trypsin (Fig. 2, track 7). After increasing the protein to rimantadine ratio up to 1 : 0.16 (Fig. 2, track 6) the potency of the drug has significantly decreased.

The inhibitory action of rimantadine is often attributed to the fact that as a weak base it accumulates in endolysosomes and alkalifies the medium (March, 1984; Beyer *et al.*, 1986), thereby preventing the conformational changes of HA. Under experimental conditions described in this paper we never observed any changes in the pH of buffer system after addition of rimantadine (up to 170 μg/ml). The protein to rimantadine ratio appeared to be of importance. Thus, for example, it can be seen in Fig. 3 that with equal rimantadine concentration (30 μg/ml) but varying quantity of the virus protein in the sample the effect of rimantadine varied significantly. At protein/rimantadine ratio 1.5 : 1 (15 μg of virus + 10 μg of rimantadine) the drug decreased tryptic digestion of the polypeptide HA1 (track 4 as compared to track 3). At protein to rimantadine ratio of 6 : 1 (60 μg of virus + 10 μg of rimantadine), the latter had no effect (track 5). At least 40 μg of rimantadine should be added to 60 μg of the virus (ratio 1.5 : 1) in order to achieve a noticeable inhibitory effect of the drug on tryptic digestion of HA1 (tracks 6 and 5).

Electron microscopy of influenza virus at low pH in the presence of rimantadine

Native viral particles at pH 7.5 had a well-marked and even undamaged layer of HA spikes (Fig. 4—I). Acid pH treatment of the virus led to morphological changes of spikes: they were no longer seen as clear separate structures but looked as if linked with each other (Fig. 4—II). Ruigrok *et al.* (1986) obtained analogous results. Upon addition of rimantadine to the virus followed by exposure to low pH the spikes were morphologically unchanged (Fig. 4—III) as compared to control samples, i.e. similarly as the virus at pH 7.5 in the presence of rimantadine (Fig. 4—IV).

Discussion

According to the common view on the mechanism of rimantadine (amantadine) action, it accumulates in endolysosomes and thereby prevents the acidification of its content (March, 1984; Beyer *et al.*, 1986). Therefore, it inhibits the process of membrane fusion (Daniels *et al.*, 1985). However, rimantadine inhibits haemolytic activity of the virus in the reaction with

chick erythrocytes (Ghendon *et al.*, 1986) in which it has apparently no effect on the pH of the buffer system (our data). The conclusion of Richman *et al.* (1986) should be also taken into account. They pointed out that for the antiviral action to develop, the drug should contact the virus outside the cell.

We have studied the action of rimantadine on the first event in succession of events leading to membrane fusion, namely on conformational reconstructing of HA induced by acid pH. Yewdell *et al.* (1983) demonstrated that incubation of influenza virus (type A) at acid pH in the absence of erythrocytes leads to a rapid and irreversible inactivation of their haemolytic activity. Our experiments have shown that addition of rimantadine to the virus before acidification helps to preserve its haemolytic activity.

Apparently, rimantadine stabilizes HA structure in some way preventing the effect of acidification on its conformation. The appearance of sensitivity to trypsin is a good evidence for the change in HA conformation (Skehel *et al.*, 1982). We have used in this paper both intact FPV virions and surface glycoprotein preparation in the form of rosettes. After incubation of virions or rosettes in the medium at pH 7.5 the trypsin treatment was without effect on the pattern of virus polypeptides. If both preparations have been exposed to acid pH before trypsin treatment the latter resulted in a sharp decrease in the amount of polypeptide HA1 and in the appearance of two new polypeptides: a major one with apparent mol. mass of 36,000 kD and a minor one with apparent mol. mass of 44,000 kD.

Upon addition of rimantadine to virions of acidified rosettes HA1 remains resistant to trypsin. It should be emphasized that an efficient rimantadine to protein ratio should be selected; the virus protein to rimantadine ratio 1 : 1 appeared optimal in our experiments. The effects on surface glycoproteins isolated from the virus have shown that the action of rimantadine on HA structure is direct and not mediated by another protein present in the virion.

Control experiments with bovine serum albumin and with proteins M and NP of FPV have demonstrated that rimantadine is without effect on the enzyme activity of trypsin (data not shown). Electron microscopic examinations of whole virions (Fig. 4) confirmed that rimantadine prevents morphological changes of surface spikes induced by acid pH. Thus, the results obtained indicate that owing to the labile binding with HA, rimantadine stabilizes its structure and prevents the acid-pH-induced conformational change which is the first in the succesion of events leading to membrane fusion.

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Explanation to Figures (Plates XVIII—XX):

Fig. 2. Electrophoresis in 10 % polyacrylamide gel of virions and surface FPV glycoproteins incubated at different pH and treated with trypsin.
Original glycoprotein (5) and virion (10) FPV preparations; glycoproteins (1) and virions (9) incubated in ST buffer pH 7.5 and treated with trypsin; glycoproteins (4) and virions (8) exposed for 20 min at 30 °C to pH 5.2, pH made neutral and the samples treated with trypsin; 10 μg of rimantadine was added to 20 μg glycoproteins (2) and/or 60 μg virions (6) in ST buffer, pH reduced to 5.2, the samples incubated for 20 min at 33 °C, neutralized and treated with trypsin; 20 μg and 60 μg of rimantadine were respectively added to 20 μg of glycoproteins (3) and 60 μg of virions (7) in ST buffer, pH reduced to 5.2, the samples incubated for 20 min at 33 °C, neutralized and treated with trypsin.

Fig. 3. Effect of protein to rimantadine ratio on trypsin resistance of HA1.
Lanes: 1 — trypsin, 2 — virus incubated at pH 7.5 and treated with trypsin; 3 — virus incubated at pH 5.2, pH made neutral and the samples treated with trypsin; 4—10 μg of rimantadine were added to 15 μg of the virus in ST buffer; pH was then reduced to 5.2, after incubation pH was made neutral and the samples were treated with trypsin; 8—10 μg of rimantadine were added to 60 μg of the virus and treated as indicated for track 4; 6—40 μg of rimantadine were added to 60 μg of the protein and then treated as indicated for track; 7 — original virus. The analysis in 10 % PAGE was carried out under reducing conditions. An equal quantity (15 μg) of the virus protein was applied on each track.

Fig. 4. Electron photomicrographs of FPV after incubation at pH 7.5 (I) and 5.2 (II); virions treated with rimantadine at pH 7.5 (III); virions treated with rimantadine and then exposed to pH 5.2 (IV).
Negative staining with phosphotungstic acid; magnified 99, 000×.