CHARACTERIZATION OF THE VIRIONS OF MOPYRIDONE-SENSITIVE WILD STRAIN AND MOPYRIDONE-RESISTANT MUTANT OF INFLUENZA VIRUS A(H3N2)

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Summary. – Some differences were established between mopyridone-sensitive (MCU-s) wild strain and mopyridone-resistant (MCU-r) mutant progenies of influenza virus A/Hong Kong/1/68 (H3N2). The virions of MCU-r mutant had a lower buoyant density in linear sucrose gradient as compared to those of MCU-s strain, and an increased ability of aggregation as well. HA content (HAU/μg protein) in the purified virions of MCU-r mutant was twice lower as compared to MCU-s strain. The surface glycoproteins of MCU-r mutant were solubilized by octylglucoside faster than those of MCU-s strain. No differences were found between MCU-r strain and MCU-s mutant-induced red blood cell lysis at acid pH, and mopyridone did not influence this phenomenon. MCU-r mutant showed a lower thermostability as compared with MCU-s strain, but similar UV-inactivation curves for both viruses were observed. The quantity of the purified HA-NA complex and M1 protein incorporated into multilamelar liposomes was greater in the case of MCU-s mutant. Electron microscopy examination of liposomes which contained M1 protein from MCU-r mutant manifested pleomorphism with unusual gigantic forms tending to aggregate, whereas MCU-s M1 protein-containing liposomes were uniform and did not form aggregates. No morphological differences were found between the two viruses in HA-NA complex containing liposomes. These data indicate changes in the protein-lipid interactions in MCU-r mutant virions. Amino acid analysis of M1 protein revealed significantly lower content of asparagine, glutamine and serine, and a higher one of histidine in MCU-r mutant as compared to MCU-s wild strain.

Key words: influenza virus A(H3N2); mopyridone resistant mutant; M1 protein; liposomes; thermostability; UV inactivation; electron microscopy

Introduction

Mopyridone, 1-(4-morpholinomethyl)-tetrahydro-2(1H)-pyrimidinone, originally synthesized in Bulgaria (Sidzhakova *et al.,* 1982), is an orthomyxo- and togavirus replication inhibitor (Galabov *et al.,* 1984), markedly effective in experimental infections in mice (Galabov *et al.,* 1994b). A detailed study of its effect on influenza virus A/Hong Kong/1/68 (H3N2) resulted in two MCU-r mutants selected from the MCU-s wild strain. Comparative analysis of the wild strain and its MCU-r mutants by solid-phase ELISA using a panel of monoclonal antibodies (MoAbs) showed some differences between M1 protein antigenic sites 1A, 2 and 3 (Galabov *et al.,* 1994a).

Some physico-chemical properties of the virions of MCU-s wild strain and MCU-r mutant of influenza virus A/Hong Kong/1/68 (H3N2) were studied in this work. Differences were found between MCU-s and MCU-r viruses in the virion density, glycoproteins (HA + NA) solubilization degree, thermostability, amino acid content of M1 pro-

Abbreviations: BSA = bovine serum albumin; ET_{99} = effective time; HA = haemagglutination; HAU = HA unit; MCU-r, MCU-s = MCU-resistant, -sensitive; MoAb = monoclonal antibody; NA = neuraminidase; PBS = phosphate buffered saline; UV = ultraviolet

tein, amount of M1 protein incorporated into liposomes, as well as in the form and size of M1 protein-containing liposomes.

Materials and Methods

Virus. Influenza virus A/Hong Kong/1/68 (H3N2), MCU-s wild strain, supplied by the National Influenza Center, Sofia, and its MCU-r mutant A/HK/1/68 (MCU-r, 3 mg (CE) 2/90) were grown in 10 day-old chick embryos.

Virus titration. The infectivity titers of virus samples were determined in chick embryonated eggs (EID_{50}) or in MDCK cell monolayers either by counting plaques (PFU) on 24 well-microplates (Falcon) or by reading CPE ($CCID_{50}$) on 96 well-microplates (Flow) by standard methods (Galabov *et al.*, 1994a). The HA titers were assayed by the routine procedure.

Virus purification. The procedure of Johansson *et al.* (1989) was used. Virion density was determined by 2 hrs centrifugation of a sample on a linear 10 – 60% (w/w) sucrose density gradient in Beckman L8 centrifuge (rotor SW11). Samples were fractionated by LKB fraction collector and their absorbance at 252 nm (A_{252}) was recorded. Both refraction index (Carl Zeiss Jena refractometer) and haemagglutination (HA) titer (HAU/ml) of each fraction were determined.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Both the number and molecular mass of viral structural proteins were determined by the Laemmli (1971) procedure using 10% polyacrylamide gel. Bovine serum albumin (BSA), ovalbumin, pepsin, chymotrypsinogen, ß-lactoglobulin and lysozyme (Sigma) were applied as standards.

Viral glycoproteins solubilization. Equal protein amounts of MCU-s and MCU-r viruses were solubilized with 2% octylglucoside (Serva). After 30 mins the suspension turbidity at 400 nm was measured. Nucleocapsids and M1 protein were separated by centrifugation at 11 000 rpm for 10 mins and protein content in supernatant was determined.

Proteins were assayed by the method of Lowry *et al.* (1951).

Turbidity was determined spectrophotometrically at 400 nm.

M1 protein was isolated by the method of Gregoriades (1973).

Amino acid content of M1 protein was determined by the aminoanalyzer Durrum Marck.

Red blood cell lysis at acid pH was done by the procedure described by Ghendon *et al.* (1986).

Thermostability test. Experiments were performed at 42 °C, 50 – 51 °C and 56 °C. The residual infectivity was determined and the 99% effective time (EI_{99}), reducing the infectivity by 99% was evaluated.

UV sensitivity test. Virus-containing suspensions were irradiated by UV-rays source (BLM-12 lamp Medicor, Budapest, 15W, lambda = 253.7 nm) from a distance of 9 cm. Residual infectivity was assayed and ET_{99} evaluated.

Liposomes were prepared using a mixture of phosphatidylcholine (Sigma) solubilized in chlorophorm-methanol (2:1, v/v) and M1 protein or HA-NA complex at a protein/lipid ratio 1:20 (w/w). Organic solvents were evaporated in a vacuum rotor evaporator. The dried film formed was resuspended in 0.5 ml distilled water by 60 mins of shaking at 37 °C. Then, 0.5 ml PBS was added and the suspension obtained was sonicated at a frequency 50 kHz for 2 x 2 mins in ultrasonicator MSE. Liposomes formed were purified by density gradient centrifugation on linear gradient of 5 – 30% sucrose (w/w) for 2 hrs at 27 500 rpm in Beckman L8 ultracentrifuge (rotor SW 28).

Electrom microscopy. Negative staining was performed with 2% sodium phosphotungstate pH 6.8. Electron microscope JEOL 1200 EX with accelerated voltage 80 kV and instrumental magnification 30 000 – 70 000 x was used.

Results

Buoyant density of virions

Data of the meassurement of buoyant density of MCU-s and MCU-r virions are shown in Fig. 1. It is obvious that MCU-r virions have a lower density in sucrose gradient as compared to MCU-r virions, 1.16 and 1.18 g/cm³, respectively. Moreover, it is well demonstrated that MCU-r virions form aggregates within the 1.18 – 1.22 g/cm³ density zone. The value of HAU/µg protein ratio was two times higher for MCU-s than for MCU-r. However, the values of HAU/EID_{50} or HAU/PFU ratios were 17 and 12 times, respectively, higher for MCU-s than for MCU-r (Table 1).

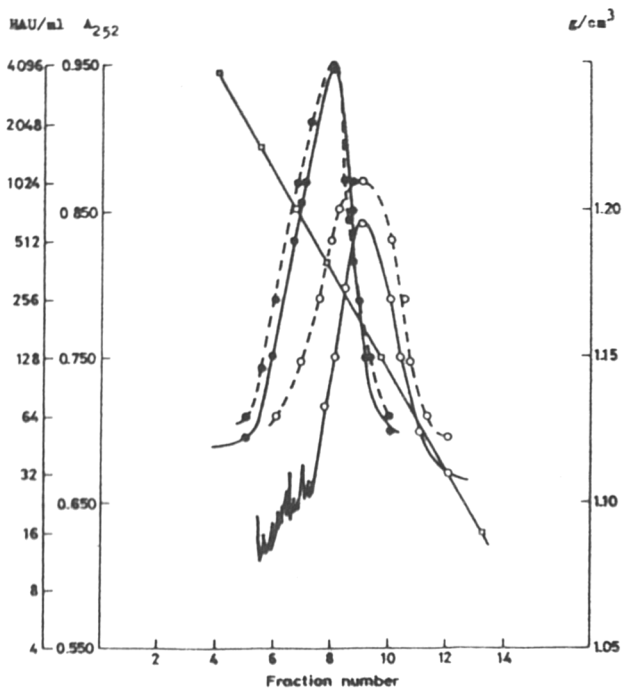

Fig. 1
Sucrose density gradient centrifugation of MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus
Ordinate: titer in HAU/ml (broken line), A_{252} (continuous line), MCU-s (●), MCU-r (○). Buoyant density in g/cm³ (continuous line, □).

Table 1. HA content in MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus

Virus	HAU/μg protein [a]	HAU/EID_{50}	HAU/PFU
MCU-s	32 770	2.05×10^{-3}	2.5×10^{-4}
MCU-r	16 200	0.12×10^{-3}	0.2×10^{-4}

[a]Purified virus.

Table 2. Solubilization with octylglucoside of surface glycoproteins of MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus

Virus	A_{400} Time (mins) 0	30	Solubilization %	Protein content (mg/ml) Time (hrs) 0	24	Solubilization %
MCU-s	1.734	1.461	15.74	2.5	0.5	20.0
MCU-r	1.734	0.940	45.79	2.5	1.04	41.6

Viral structural proteins

Analysis of the structural proteins of MCU-s wild strain and MCU-r mutant by SDS-PAGE demonstrated no differences in both the number of various proteins and their molecular mass in the two mutants (Fig. 2).

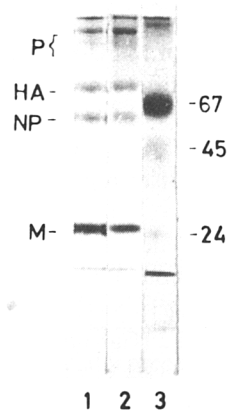

Fig. 2
SDS-PAGE of virion proteins of MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus
MCU-s (lane 1), MCU-r (lane 2), reference proteins BSA, serum albumin, ovalbumin, pepsin, chymotrypsinogen, ß-lactoglobulin and lysozyme (lane 3).

Viral glycoproteins solubilization

Detergent treatment of influenza virus particles could give an information on surface proteins linkage to lipid bilayer. Comparative data concerning the effect of octylglucoside on virions of MCU-s and MCU-r viruses are shown in Table 2. It can be seen that the glycoproteins of MCU-r mutant are solubilized more easily than those of MCU-s wild strain. The turbidity of viral particles suspension after 30 mins of solubilization was lower for MCU-r than for MCU-s. After 24 hrs of solubilization the content of solubilized MCU-r viral glycoproteins was two times higher than that of MCU-s.

Influence of mopyridone on red blood cell lysis induced by MCU-s wild strain and MCU-r mutant at acid pH

Viral-induced haemolysis at acid pH is a precise method to substantiate changes in influenza virus HA. It was used to characterize HA as target of viral inhibitors (Ghendon *et al.*, 1986). We studied the mopyridone effect on haemolytic activity of MCU-s and MCU-r viruses. Preliminary experiments showed that MCU-s wild strain as well as MCU-r mutant possess a pronounced haemolytic activity at pH 5.0 – 5.2. Further experiments demonstrated (Table 3) that mopyridone does not affect the haemolytic activity at pH 5.2 of both MCU-s and MCU-r viruses, even at concentrations of 100 μg/ml and 200 μg/ml.

Thermostability

Physico-chemical characteristic of MCU-s and MCU-r viral particles was supplemented with a study of their

Table 3. Influence of mopyridone on red blood cell lysis induced by MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus at acid pH

Mopyridone concentration (μg/ml)	A_{520} Blank sample	Virus MCU-s	MCU-r
0	0.090	0.386	0.352
100	0.081	0.322	0.392
200	0.092	0.403	0.354

Five to ten μg of purified virus in 200 μl of MM buffer pH 7.0 (0.005 mol/l Tris-HCl, 0.14 mol/l NaCl) was mixed with 300 μl of 8% chick red blood cells, MCU was added, and the mixture was incubated at room temperature for 20 mins, and at 0 °C for 10 mins. Then citrate buffer pH 5.2 was added, the mixture incubated at 37 °C for 30 mins with periodic stirring, and A_{520} read. Blank sample contained no virus.

Table 4. Thermostability and UV sensitivity of MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus

Virus	ET_{99}			
	42 °C	51 °C	56 °C	UV irradiation
MCU-s	40 mins 45 secs	30 mins 0 secs	5 mins 0 secs	2 mins 48 secs
MCU-r	20 30	17 30	3 12	2 30

ET_{99}, 99% effective time, in which viral infectivity was reduced by 99% (infectivity titer reduced by 2 log units). ET_{99} was evaluated from virus infecting inactivation curves (log $CCID_{50}$/ml versus time). Virus infectivity was titrated on MDGK cells in 96 well-microplates.

thermostability at 42 °C, 50-51 °C and 56 °C (Table 4). It was found that MCU-r mutant has a markedly lower thermostability as compared to the MCU-s wild strain.

Sensitivity to UV irradiation

No difference was found between MCU-s and MCU-r viruses in their sensitivity to UV irradiation (Table 4).

Liposomes with incorporated influenza virus A structural proteins

HA-NA protein complex and M1 protein isolated from MCU-s and MCU-r viruses were incorporated into liposomes prepared with phosphatidylcholine. Values of buoyant density and protein content of liposomes are illustrated in Table 5. A slight decrease in incorporation of both HA-NA and M1 from MCU-r mutant as compared to MCU-s wild strain is seen. However, the density of MCU-r M1 containing liposomes was slightly higher than that of MCU-s M1 ones.

Electron microscopy of liposomes

Electron microscopy examination of liposomes with M1 protein from MCU-r mutant manifested pleomorphism with unusual gigantic forms tending to aggregate (Fig. 3, d-f). MCU-s M1 protein containing liposomes were uniform and did not form aggregates (Fig. 3, a-c). In contrast, no differences were observed between the two viruses in HA-NA complex containing liposomes (data not shown).

Table 5. Characterization of liposomes with incorporated HA-NA or M1 protein of MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus

Virus	Virus protein incorporated	Protein content (μg/ml)	Incorporated protein[a] (%)	Liposome buoyant density (g/cm^3)
MCU-s	HA-NA	140	27	ND
	M1	66	26	1.08
MCU-r	HA-NA	110	21	ND
	M1	40	16	1.10

ND = not done. [a]Calculation based on the amount of purified HA-NA or M1 protein added to the lipid mixture for liposome formation.

Amino acid content of M1 protein

Table 6 demonstrates a significantly lower content of asparagine, glutamine and serine, and a higher content of histidine in M1 protein of MCU-r mutant as compared to MCU-s wild strain. No differences were found in the content of other amino acids.

Discussion

Our previous study has demonstrated significant changes in the structure of M1 protein of MCU-r mutant of influenza virus A/Hong Kong/1/68 (H3N2), more precisely in antigenic sites 1A, 2 and 3 (Galabov *et al.*, 1994a).

Table 6. Amino acid content of M1 protein isolated from MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus

Amino acid	MCU-s		MCU-r	
	Quantity (nmol/l)	Percentage[a]	Quantity (nmol/l)	Percentage[a]
Asp	6.2	11.9	4.6	10.7
Thr	3.4	6.5	2.6	6.0
Ser	5.3	10.1	3.9	9.0
Glu	8.9	17.0	6.5	15.0
Gly	4.6	8.8	3.6	8.3
Ala	5.9	11.3	4.6	10.7
Val	2.8	5.4	2.4	5.5
Ile	2.0	3.8	1.9	4.4
Leu	5.5	10.5	4.5	10.4
Tyr	1.2	2.3	1.1	2.5
Phe	1.9	3.6	1.8	4.1
Lys	3.2	6.1	2.8	6.5
His	1.6	3.1	1.9	4.4

[a]Number of residues of given amino acid per 100 amino acid residues.

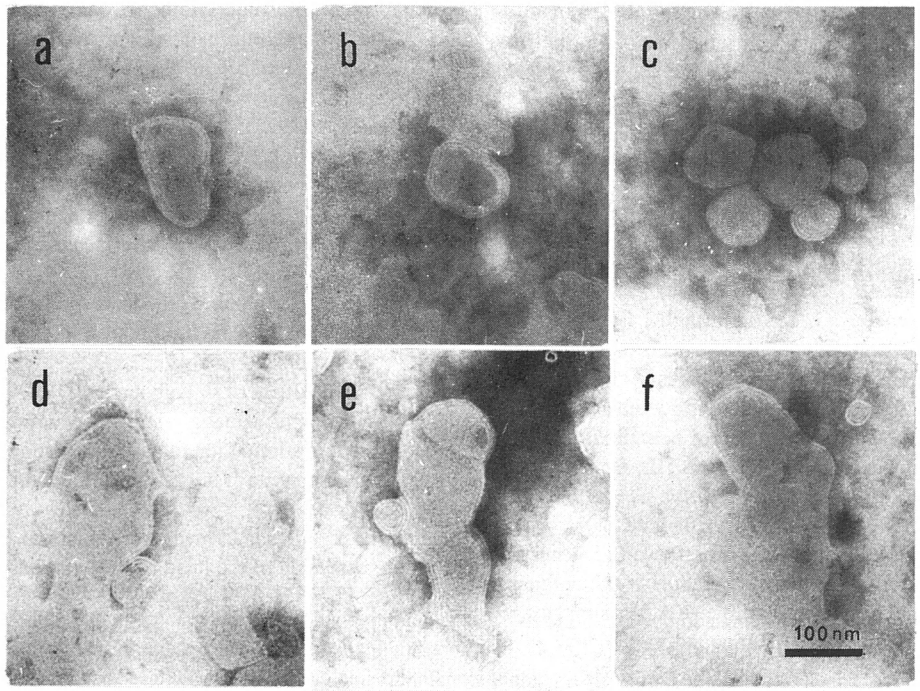

Fig. 3
Electron micrographs of sucrose gradient-purified liposomes formed in the presence of M1 protein of MCU-s wild strain and MCU-r mutant of influenza A(H3N2) virus
Liposomes with M1 protein of MCU-s wild strain (a,b,c) and MCU-r mutant (d,e,f).

It is known that influenza virus M1 protein plays a key role in the virion assembly and structure stability (Schulze, 1970). This protein has a high affinity for lipids and is easily incorporated into arteficially prepared lipid vesicles (Bucher *et al.,* 1980; Gregoriades, 1980). Interaction of M1 protein with the lipid bilayer could alter the membrane structure during viral replication and thus result in changes of viral glycoprotein spikes (Davies and Bucher, 1981). Obviously, alterations in M1 protein structure could provide a basis for changes in M1 protein-lipid interactions and virion stability.

Our experimental results reported here on some properties of the virions and M1 protein of influenza virus A(H3N2) mopyridone mutant MCU-r in comparison to MCU-s wild strain are in correspondence with the data reviewed above. Thus, we clearly demonstrated that MCU-r M1 protein-containing liposomes had an unusual gigantic form and a lower protein content as compared to MCU-s. The M1-specificity of this phenomenon is demonstrated by the lack of differences between MCU-s and MCU-r viruses. Moreover, the faster solubilization of MCU-r glycoproteins could also be connected with changed protein-lipid interactions in the virions.

The lower HAU content in MCU-r virus particles with being preserved infectivity could be due to the altered virion structure, too. It is not likely that there are involved changes in HA protein of MCU-r as the test with red blood cell lysis at acid pH demonstrated no differences between MCU-s and MCU-r viruses. Besides, no changes were observed in HA antigenic structure of MCU-r mutant by use of MoAbs (A.S. Galabov, unpublished data).

As concerns the heat and UV tests, our data manifested a lower virion stability of MCU-r mutant and a lack of polygenomic viral particles in both MCU-r and MCU-s viruses. However, the purified virions as well as the M1 containing liposomes of MCU-r mutant revealed a tendency to aggregate, a phenomenon due probably to some alterations in the protein hydrophobicity. This suggestion could be substantiated by the changes found in the content of some polar amino acids (asparagine, glutamine, histidine) in MCU-r M1 protein.

Our data on a decreased amount of asparagine and glutamine in M1 protein of MCU-r mutant are in accordance to those of Gregoriades and Frangione (1981) on the obligatory presence of these amino acids in the sites of M1 protein binding to liposomes.

In summary, data presented here support our hypothesis on M1 protein as a target of anti-influenza virus effect of mopyridone.

There are reasons to assume that antiviral substances with a mode of action directed to M1 protein may be considered

useful not only for chemotherapeutic aims, but also in studies on the role of M1 protein in influenza virus replication.

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