VIROLOGY

Comparative Study of Reproduction of Sindbis Virus Sensitive and Resistant to Adamantane Derivatives

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The sensitivity of Sindbis virus gradually decreases during serial passages in the presence of 1-adamantane carbonic acid amide. After 4 passages, viral reproduction was not suppressed even by subtoxic concentrations of the inhibitor. Sensitive Sindbis strain principally differed from the resistant one: the latency of reproduction of resistant virus was 2 h longer than that of the sensitive strain. Cells infected with the sensitive and resistant strains of Sindbis virus contained the major structural proteins (C, E1+E2), their precursors pE2, p130, p90, and p78 protein, which probably represent a nonstructural virus-specific protein nsP3. The synthesis of virus-specific polypeptides and RNA of resistant variant is resistant to the inhibitor during incubation.

Key Words: *Sindbis virus; antiviral substance; resistant variant; characteristics of resistant variant*

It is well known that virus strains resistant to any antiviral drug can be obtained. Amantadine- and remantadine-resistant strains of influenza virus, acyclovir-resistant strains of herpes simplex and varicella zoster viruses, azidothymidine-resistant strains of human immunodeficiency virus, and gancyclovir-resistant strains of human cytomegalovirus have been described [8,11-14]. The presence of these strains in virus populations can reduce the efficiency of drug therapy. However drug resistance of alphaviruses has virtually never been studied.

It has been shown that some adamantane derivatives effectively inhibit reproduction of Sindbis neurovirus (SV). Serial passages of SV with 1-adamantane carbonic acid amide (ACAA), the most potent antiviral drug, resulted in derivation of resistant SV variant [1]. In this study we compared characteristics of resistant and original sensitive variants of the virus.

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MATERIALS AND METHODS

A 36-h Vero cell culture (Tissue Culture Laboratory of Institute of Virology) was used. SV strain Ag 339 was from Virus Museum Laboratory of the Institute. Resistant SV strain was obtained by serial passages with ascending ACAA concentrations [1].

ACAA was synthesized at Polytechnical Institute (Kiev) and kindly given by Prof. A. G. Yurchenko and Dr. S. D. Isaev.

Antiviral activity of the test compounds was evaluated routinely by the decrease in infective titer [1]. The development of virus-specific cytopathic effect in infected cells was evaluated using trypan blue staining [1] (multiplicity of infection (MI) of plaque-forming units (PFU)/cell).

RNA synthesis in infected cells was studied as described previously [15]. The cells were incubated with 5 μCi/ml ^{3}H-uridine for 1 h and its incorporation in acid-insoluble cell fraction was evaluated. Cellular RNA synthesis was inhibited with 5 μg/ml actinomycin D (Calbiochem). Multiplicity of infection was 50 PFU/cell.

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Proteins were analyzed by electrophoresis in polyacrylamide gel (PAAG) as described elsewhere [10]. After 5-h incubation of cells (MI 50 PFU/cell), protein was labeled with ^{35}S-methionine (50 μCi/ml) for 1 h. Cell monolayer was lyzed as described previously [10]. Actinomycin D (2 μg/ml) was used at all stages of the experiment for inhibition of protein synthesis.

The results were processed statistically using routine methods [2].

RESULTS

ACAA is an effective selective inhibitor of SV reproduction [1], it effectively protects cells from death in a wide range of concentrations: for CD_{50} ACAA is 191.25 μg/ml and ID_{50}=11.25 μg/ml (for remantadine CD_{50} 51.25 μg/ml and ID_{50}=21.25 μg/ml; CD_{50} is the concentration of the compound at which 50% cells survive and ID_{50} is the concentration protecting 50% infected cells from death).

Study of virus accumulation in the culture medium showed that ACAA in a concentration approximating 1/2 CD_{50} (100 μg/ml) decreased the infective titer of the virus by 6 lg PFU/ml (MI 0.01 PFU/cell) and more (Table 1), while remantadine in a concentration 50 μg/ml (CE_{50}) decreased it only by 2.2 lg PFU/ml. Chemotherapeutic index of the test compound calculated as the ratio of CD_{50} to the minimum active concentration (MAC; concentration decreasing the infective titer of the virus by at least 1.25 lg PFU/ml) is 30.6, which indicates a pronounced selective action of ACAA.

Serial passages of SV with ACAA in a concentration gradient of 50-100 μg/ml resulted in selection of a ACAA-resistant strain (ACAA-R) [1].

The sensitivity of SV and its passages to ACAA in concentrations 25-200 μg/ml is presented in Table 1. The sensitivity of SV to ACAA in higher concentrations was not studied, because they are toxic for cells.

SV clones resistant and sensitive to ACAA (ACAA-R and ACAA-S) were isolated from plaques extracted from agar. The study macromolecule synthesis (RNA and proteins) in cloned SV showed reduced sensitivity of total RNA production by ACAA-R SV to ACAA in comparison with ACAA-S. Six and 8 hours after cell infection with ACAA-S and ACAA-R, respectively, when ^{3}H-uridine incorporation into acid-insoluble cell fraction is maximum [1], 50% inhibition of ^{3}H-uridine incorporation in acid-soluble fraction of these cells was attained in the presence of 22.5 and 162.5 μg/ml ACAA, respectively.

ACAA selectively inhibited reproduction of Venezuelan equine encephalomyelitis (VEE) virus, another alphavirus, in Vero cells in the same concentrations as for SV: ID_{50}=10.64 μg/ml and chemotherapeutic index equal to 18 [1]. In a concentration of 100 μg/ml ACAA decreased the infective titer of VEE virus by 6.48 lg PFU/ml (MI 0.01 PFU/cell). ACAA effectively decreased the infective titer of VEE virus in the brain of 10-12-g white mice after intracerebral inoculation. The scheme of oral administration of ACAA (single dose 150 mg/kg) is as follows: 4 h before and 4 h after infection, than a single dose after 1, 3, 5, and 7 days. As was shown previously with SV, this is an optimal protocol reducing the titer by 4-lg. *In vitro* culturing of VEE virus with ascending concentrations of ACAA (starting from 50 μg/ml in the first passage to 100 μg/ml in the fourth) leads to the formation of a virus population highly resistant to ACAA and its derivatives: the infective titer does not decrease in the presence of 100 μg/ml ACAA.

We evaluated synthesis of virus-specific SV polypeptides in the presence of ACAA immediately after infection. All virus-specific polypeptides were present in infected cultures (Fig. 1, *4*): capsid protein C (29 kD), envelope glycoproteins E1 and E2 (50+50 kD), E2 glycoprotein precursor pE2 (62 kD), and minor components p130, p90, and p78 which are absent in intact cells (Fig. 1, *2*); this is in line with published reports [3,7].

TABLE 1. Infective Titers (lg PFU/ml) of SV Variants

ACAA concentration, μg/ml	Parent	SV after passage			
		1	2	3	4
0	8.12	7.57	8.12	8.06	7.84
25	5.89	5.70	7.38	8.08	7.96
50	4.02	4.26	5.81	7.83	8.00
100	2.10	2.96	5.08	6.45	7.55
150	1.64	—	—	—	6.90
200	0.62	—	—	—	6.57

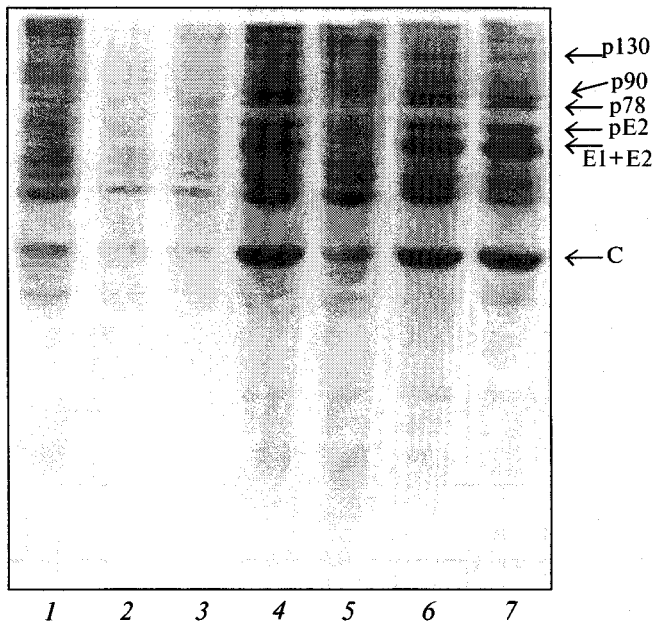

Fig. 1. Radioautograph of PAAG electrophoregram of Sindbis virus polypeptides (MI 50 PFU/cell; concentration of 1-adamantane carbonic acid amide (ACAA) 100 μg/ml, actinomycin D 2 μg/ml). Intact cells (*1*), incubated with actinomycin D (*2*); with actinomycin D and ACAA (*3*). Cells infected with ACAA-sensitive Sindbis virus incubated without (*4*) and with ACAA (*5*). Cells infected with ACAA-resistant Sindbis virus incubated without (*6*) and with ACAA (*7*).

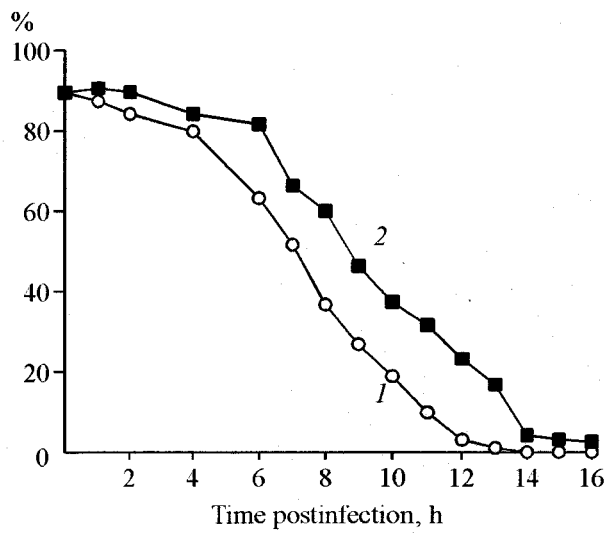

Fig. 2. Time course of virus-specific cytopathic effect in Vero cells infected with Sindbis virus sensitive (*1*) and resistant (*2*) to 1-adamantane carbonic acid amide. (Trypan blue staining, MI 10 PFU/cell). Ordinate: number of viable cells, %. *1*) ACAA-S; *2*) ACAA-R.

ACAA partially inhibited the synthesis of virus-specific polypeptides in infected cells (Fig. 1, *5*). ACAA did not prevent the synthesis of virus-specific polypeptides in cells infected with ACAA-R SV (Fig. 1, *6, 7*). The results in samples 4 and 6 are virtually the same.

Plotting the SV growth curve (MI 10 PFU/cell) and studying total SV RNA synthesis in the presence of actinomycin D (MI 50 PFU/cell) we showed that the latency of ACAA-R SV increased by 2 h in comparison with ACAA-S [1]. Virus-induced cytopathic effect in cell cultures infected with ACAA-R SV (Fig. 2, *2*) attained 97.4% only after 16-h incubation, while in cell cultures infected with ACAA-S SV (Fig. 2, *1*) no viable cells were found after 14 h. Evidently, the reproductive cycle of ACAA-R SV is prolonged in comparison with ACAA-S due to increased latency.

An SV mutant (strain AR 339) with a 3-h shorter latency in comparison with the parent virus due to Ser-114→Arg substitution in glycoprotein E2 has been described [6]. Asn-62→Asp substitution in E2 leads to reversion of this sign [13]. Hence, glycoprotein E2 contains a domain including Ser-114 and Arg-62 determining the rate of virus penetration into the cell. Probably, prolonged latency in ACAA-R SV obtained by us is caused by mutation(s) in the same domain of glycoprotein E2. Previously demonstrated cross-resistance of ACAA-R SV [1] to weak lysosomotropic bases ammonium chloride and remantadine, increasing pH of endosomal contents [9], indirectly confirms this conclusion. Fusion activity of SV penetrating into the cell via receptor-mediated endocytosis is due to surface spikes formed by glycoproteins E1 and E2. The resistance of ACAA-R SV to remantadine and ammonium chloride can result from activation of its fusion protein at higher pH values. This can be due to amino acid substitutions in E2 affecting the rate of SV penetration into the cell and modifying migration of the respective polypeptides in PAAG. For instance, PAAG migration of hemagglutinin from remantadine-resistant influenza virus responsible for its fusion activity differ from that of sensitive virus [4,5]. However comparative electrophoresis of virus-specific polypeptides from ACAA-S and ACAA-R SV (Fig. 2, *4* and *6*, respectively) showed no differences between these variants.

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