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EVALUATION OF ANTIVIRAL SUBSTANCES AGAINST INFLUENZA A VIRUS STRAINS BY THE HAEMADSORPTION REDUCTION TEST

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Summary. — The haemadsorption reduction test with Ehrlich mouse ascites tumour cells proved to be a simple method for testing the sensitivity of influenza A virus strains to antiviral substances. Ribavirin and 2-deoxy-D-glucose were poorly effective in this system while all virus strains except A/PR/8/34 (H0N1) were highly sensitive to amantadine and rimantadine, the latter being the strongest inhibitor.

Key words: *influenza virus*; *antiviral substances*; *haemadsorption*

Introduction

Due to the continuous variations in the antigenic properties of influenza viruses known as shift and drift, an epidemiological control of the virus solely by vaccination does not appear feasible. Therefore, chemoprophylaxis and chemotherapy play an important role in the combat of influenza epidemics. This requires the development of test evaluating the effectiveness of drugs and the drug-sensitivity of viruses.

The plaque reduction test (Appleyard, 1977) and the haemadsorption test employed by Schild and Sutton (1965) require the use of monolayer cell cultures and are therefore relatively labour- and time-consuming. In addition, not all influenza strains form plaques.

In extension of the experiments of Adamczyk (1977) with amantadine, we tested four substances (amantadine, rimantadine, ribavirin and 2-deoxy-D-glucose) for their antiviral effect against influenza A virus strains by the haemadsorption reduction technique on mouse ascites tumour cells.

Materials and Methods

Virus strains. Eleven influenza A virus strains were tested (see Table 1).

Cells. Suspension cultures of fresh Ehrlich mouse ascites tumour cells were used. The culture medium consisted of phosphate buffered saline (PBS; Dulbecco and Vogt, 1954) with 0.5% yeast extract, phenol red as indicator, penicillin (100 I. U./ml), streptomycin (100 μg/ml) and mycostatin (100 I. U./ml).

Antiviral substances. Amantadine (Viregyt^R; Egyt Pharmacochemical Works, Budapest, Hungary) and 2-deoxy-D-glucose (Reanal, Hungary) was used. Rimantadine was generously

Table 1. Sensitivity of influenza A virus strains to amantadine and rimantadine

Virus strain	Number of haemadsorbing cells per ml (log_{10})								
	Con-trol	Amantadine (μg/ml) 100	10	1.0	0.1	Rimantadine (μg/ml) 100	10	1.0	0.1
A/PR/8/34(H0N1)	5.07	4.54	4.93	4.93	4.95	0	5.05	5.27	5.15
A/New Jersey/8/76(Hsw1N1)	5.23	4.11	4.52	4.52	4.80	0	4.60	4.54	4.60
A/FM/1/47(H1N1)	5.48	3.00	4.65	4.76	5.00	3.00	4.54	4.67	4.72
A/Berlin/18/78(H3N2)	5.46	4.65	5.03	5.09	5.29	3.00	4.81	4.81	4.99
A/Hong Kong/8/68(H3N2)	5.18	3.00	3.95	4.49	4.95	0	3.70	4.36	4.65
A/Berlin/4/78(H1N1)	4.91	0	3.70	3.78	4.28	3.00	3.00	3.85	4.18
A/Berlin/20/78(H1N1)	4.85	3.48	4.15	4.04	4.00	0	3.70	4.00	4.04
A/Berlin/7/78(N1N1)	4.79	0	3.48	4.08	4.74	0	0	3.47	3.84
A/Berlin/13/73(H3N2)	5.51	3.30	4.48	4.86	5.19	3.00	3.30	4.86	4.91
A/Berlin/9/68(H3N2)	5.00	0	3.70	4.00	4.59	3.00	3.30	3.85	4.08
A/Berlin/1/64(H2N2)	4.77	3.00	3.48	3.85	4.53	0	3.30	3.30	3.90

0 means no haemadsorbing cells.

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The haemadsorption reduction test was performed according to Adamczyk *et al.* (1975). Mouse ascites tumour cells diluted in culture medium to a concentration of 4×10^6 cells/ml were incubated with an equal volume of a solution of the respective substance at the double final concentration for up to 2 hr at room temperature. The cells were then infected with influenza virus-containing allantoic fluid at a multiplicity of about 0.05 infective particle per cell. After at least 16 hr of incubation at 37 °C, a portion was treated for about 2 hr at 4 °C with a 0.5% guinea pig erythrocyte suspension. The haemadsorbing cells were counted in a Fuchs-Rosenthal chamber. Inhibitor-free samples served as controls. The significance of differences in haemadsorbing cell counts was tested in advance: a difference of 0.5 log unit per ml was still statistically significant (5% probability of error). Under our experimental conditions none of the substances at the concentrations applied showed any cytotoxic effect as compared with the rate of cell proliferation of untreated cells.

Results

Table 1 shows that significant levels of inhibition occurred at amantadine and rimantadine concentrations as low as 1 or 0.1 μg/ml, respectively, while ribavirin and 2-deoxy-D-glucose only inhibited virus growth at the highest concentration applied, 100 μg/ml (Table 2). In contrast to ribavirin and 2-deody-D-glucose which influenced the growth of all virus strains to the same extent, the strains responded differently to rimantadine and amantadine. The prototype strain A/PR/8/34 (H0N1) belonged to the group of relatively insensitive influenza A virus strains, the growth of which was only affected at the highest inhibitor concentration. The other group comprising the majority of influenza A virus strains was characterized by a sensitivity to even very low amantadine and rimantadine concentrations (down to 0.1 μg/ml). Representatives of the latter group were A/Berlin/4/78 (H1N1), A/Berlin/20/78 (H1N1) and A/New Jersey/8/76 (Hsw1N1).

Table 2. Sensitivity of influenza A virus strains to ribavirin and 2-deoxy-D-glucose

Virus strain	Number of haemadsorbing cells per ml (log_{10})								
	Control	Ribavirin (μg/ml				2-Deoxy-D-glucose (μg/ml)			
		100	10	1.0	0.1	100	10	1.0	0.1
A/PR/8/34(H0N1)	5.07	4.56	5.02	5.13	5.19	0	4.91	5.11	5.08
A/New Jersey/8/76(Hsw1N1)	5.23	4.43	5.00	5.04	5.05	3.00	4.90	5.25	5.02
A/Berlin/18/78(H3N2)	5.46	5.03	5.31	5.28	5.39	3.78	5.20	5.32	5.40
A/Berlin/4/78(H1N1)	4.91	4.18	4.59	4.81	4.76	0	4.52	4.58	4.49
A/Berlin/20/78(H1N1)	4.85	4.26	4.80	4.83	4.85	0	3.60	4.74	4.75
A/Berlin/13/73(H3N2)	5.51	5.00	5.31	5.42	5.51	0	5.15	5.24	5.29
A/Berlin/9/68(H3N2)	5.00	4.36	4.81	4.85	4.88	0	4.46	4.76	4.84
A/Berlin/1/64(H2N2)	4.77	3.70	4.45	4.56	4.65	3.70	3.85	4.45	4.34

0 means no haemadsorbing cells.

Table 1 also shows that rimantadine inhibited virus growth significantly down to 0.1 μg/ml while amantadine action levelled off at 1 μg/ml. Additional experiments (data not shown) proved that, under the given conditions, rimantadine was still effective at 0.01 μg/ml.

Discussion

The present results emphasized the importance of amantadine and rimantadine as antiviral drugs for influenza prophylaxis, at the same time proving the existence of influenza strains of low sensitivity (at least in vitro), like A/PR/8/34 (H0N1). These data are in agreement with those of Appleyard (1977) and Oxford and Schild (1977). Though amantadine-resistant strains have not yet been isolated from amantadine-treated influenza patients, the appearance of resistant virus variants under natural conditions is probable (Oxford, 1974). Oxford and Potter (1973) succeeded in selecting resistant strains after passages through mice treated with high amantadine concentrations. Tučkova *et al.* (1973) and Appleyard (1977) isolated amantadine-resistant mutants from embryonated eggs and in cell culture. Recombination experiments carried out by Lubeck *et al.* (1978) with A/Hong Kong/8/68 (H3N2) and A/PR/8/34 (H0N1) influenza virus strains indicated that differences in susceptibility to amantadine among the recombinants are most closely associated with differences in the gene coding for M protein.

Presber *et al.* (1974) and Scholz *et al.* (1979) discussed the different sensitivity of virus mutants of one species towards an inhibitor as an indication of the specific action of this inhibitor towards a virus-coded protein or enzyme. These authors also proposed to develop specific screening methods on this basis. Our data could support the validity of this conception. Concerning the equally low sensitivity towards ribavirin and 2-deoxy-D-glucose of all virus strains examined there was no specific drug resistance. As described by Klenk *et al.* (1972), 2-deoxy-D-glucose is less effecttive, producing an antiviral effect only at higher concentrations.

The advantage of the haemadsorption method on Ehrlich mouse ascites tumour cells consists in the short incubation time (16 hr). Thus virus growth can be tested under one-step conditions. The method has been used for virus titration and in a modified form (haemadsorption inhibition test) it is also suitable for determining antibody and for typing unidentified virus isolates (Adamczyk *et al.*, 1975). The results of Adamczyk (1977) and the present ones showed that this method (applied as haemadsorption reduction test) is also convenient for evaluating the sensitivity of influenza viruses against antiviral substances. In pilot experiments we found the haemadsorption technique with ascites tumour cells also applicable to influenza B and C, parainfluenza and mumps viruses.

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