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OCCURRENCE OF AMANTADINE- AND RIMANTADINE-RESISTANT INFLUENZA A VIRUS STRAINS DURING THE 1980 EPIDEMIC[1])

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Summary. — The sensitivities to amantadine and rimantadine of influenza A virus epidemic strains were assayed by the haemadsorption reduction test in mouse Ehrlich ascites cells in comparison with prototype strains and a rimantadine-resistant mutant. Besides a majority of sensitive strains, two relatively resistant epidemic strains were identified. The possible origin of resistant strains and their importance for medical practice are discussed.

Key words: influenza A virus; amantadine; rimantadine; antiviral substances

Introduction

Since the use of vaccines is limited due to the antigenic variability of influenza A viruses, antiviral agents could play an important role in influenza control. The prophylactic and, if administered early after infection, also therapeutic effects of amantadine and its derivative rimantadine are known (Krylov *et al.*, 1978; Hoffmann, 1980; Junker and Scheifele, 1980). The appearance of drug-resistant virus mutants in the course of an influenza epidemic is thus of practical significance. Such mutants have previously been isolated in the laboratory by passaging the virus in the presence of amantadine or rimantadine in cell cultures, embryonated hen's eggs and mice (Tučková *et al.*, 1973; Ilyenko, 1975; Appleyard, 1979; Feldblum, 1979).

Materials and Methods

Virus strains. Twenty-one influenza A (H3N2) virus strains were isolated in Berlin from patients during the epidemic of 1980 over a period of 7 weeks (Table 1). The A/Krasnodar/101/59 (H2N2) strain is a Soviet vaccine strain; the resistant mutant was derived from it after four egg passages in the presence of 3 mg rimantadine per egg (Feldblum, 1979). The influenza proto-

[1]) Dedicated to Prof. K. Spies on the occasion of his sixtieth birthday.

Table 1. Sensitivity of influenza A/Berlin (H3N2) virus strains to amantadine and rimantadine

Strains	Date of isolation from patients (1980)	Haemadsorbing cells per ml (log_{10})							
		control	amantadine (μg/ml)			rimantadine (μg/ml)			
			10	1	0.1	10	1	0.1	0.01
highly sensitive									
8*	14.2.	4.93	4.30	4.26	4.53	3.85	3.90	3.78	4.23
23	6.2.	5.16	4.59	4.57	4.76	4.28	4.45	4.45	4.65
28	11.2.	4.96	4.23	4.36	4.76	3.95	4.23	4.20	4.36
36	12.3.	5.24	4.20	4.56	4.78	4.20	4.23	4.40	4.58
inter-mediate									
6	6.2.	5.34	4.80	4.79	5.07	4.63	4.60	4.56	4.90
17	13.2.	4.93	4.23	4.32	4.56	3.95	3.95	3.95	4.53
18	14.2.	5.28	4.77	4.76	5.09	4.76	4.85	4.76	5.01
19	15.2.	5.03	4.15	4.28	4.93	3.90	3.90	4.38	4.79
22	14.2.	5.03	4.18	4.46	4.90	4.46	4.36	4.30	4.82
24	19.2.	5.05	4.36	4.65	4.89	4.34	4.32	4.43	4.74
25	19.2.	4.67	4.15	4.38	4.57	4.04	4.17	4.32	4.54
26	22.2.	5.49	4.73	4.82	5.22	4.88	4.70	4.86	5.20
27	22.2.	4.90	4.00	4.04	4.69	4.04	4.15	4.08	4.58
29	18.2.	5.24	4.15	4.11	4.93	3.85	3.85	4.40	4.80
30	28.2.	4.66	4.00	4.15	4.38	3.60	3.78	4.23	4.26
31	3.3.	4.99	4.30	4.43	4.84	4.32	4.28	4.32	4.80
32	19.2.	5.49	4.60	4.54	5.09	4.08	4.20	4.28	5.12
33	18.2.	5.04	4.15	4.28	4.77	4.00	4.15	4.15	4.70
34	26.2.	5.76	5.14	5.17	5.62	5.10	5.18	5.16	5.47
relat. resistant									
21	5.2.	5.22	4.92	4.92	5.18	4.89	4.94	4.88	5.06
37	27.3.	5.76	5.28	5.42	5.51	5.34	5.38	5.39	5.50

* Full designation of the strains: A/Berlin/8/80 (H3N2) etc.

type strains listed in Fig. 2 were supplied by the National Influenza Reference Laboratory, Berlin, G.D.R.

Cells. Fresh mouse Ehrlich ascites tumour cells were maintained in suspension culture in a medium containing phosphate buffered saline (Dulbecco and Vogt, 1954), 0.5% yeast extract, phenol red, penicillin (100 units/ml) and streptomycin (100 μg/ml).

Antiviral substances. Amantadine hydrochloride (1-adamantane amine HCl, Symmetrel^R) was a product of SERVA (Heidelberg). Rimantadine hydrochloride (alpha-methyl-1-adamantane methylamine HCl) was synthesized in the Institute of Organic Synthesis, Latvian Academy of Sciences, Riga, U.S.S.R.

The haemadsorption reduction test was performed as suggested by Adamczyk (1977). Fresh Ehrlich ascites tumour cells (4×10^6/ml) were incubated with an equal volume of medium with or without the inhibitor for 2 hr at room temperature. The cells were then infected with influenza virus (in the form of infectious amniotic fluid) at a multiplicity of 0.05 infectious particles per cell. After 18 hr incubation at 37 °C, a 0.5% suspension of guinea pig red blood cells was added, followed by 2 hr incubation at 4 °C. Haemadsorbing cells were counted in a Fuchs Rosenthal chamber. Earlier experiments had shown that 0.5 log_{10} unit differences between the counts of treated and untreated infected cells, i.e. a reduction of haemadsorption by 70%, was statistically significant ($p < 5\%$). Under our experimental conditions both tested substances were not cytotoxic.

Results

Table 1 shows that influenza A epidemic strains differed in their sensitivity to rimantadine and amantadine. Based on these differences the strains could be divided into three groups: a) highly sensitive strains (like A/Berlin/8 /80, A/Berlin/23/80, A/Berlin/28/80, A/Berlin/36/80; b) strains of intermediate sensitivity (like A/Berlin/6/80, A/Berlin/19/80, A/Berlin/26/80, A/Berlin/

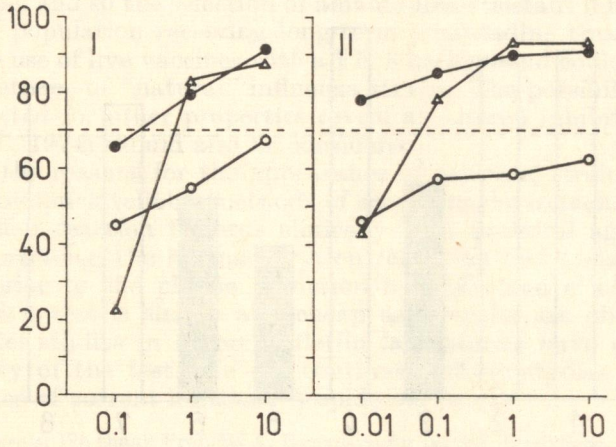

Fig. 1.

Sensitivity of influenza A epidemic strains to amantadine (I) and rimantadine (II) as determined by the haemadsorption reduction test

Area above the broken line: significant inhibition ($p < 5\%$).

● A/Berlin/36/80 (H3N2)

▲ A/Berlin/19/80 (H3N2)

○ A/Berlin/37/80 (H3N2)

Abscissa: drug concentration in μg/ml; ordinate: inhibition %

33/80); and c) relatively resistant strains (A/Berlin/21/80, A/Berlin/37/80). Highly sensitive strains were still inhibited by 0.01 μg/ml rimantadine, but not by 0.1 μg/ml amantadine; strains of intermediate sensitivity were inhibited by 0.1 μg/ml rimantadine or 1 μg/ml amantadine. The haemadsorption counts for both relatively resistant strains were not affected by even the highest inhibitor concentrations employed (Fig. 1).

Rimantadine was about 10 times as effective as amantadine, exerting inhibition at concentrations as low as 0.1 to 0.01 μg/ml (Table 1). The rimantadine-resistant mutant was about as insensitive as the epidemic strains and comparable to the laboratory strain A/PR/8/34 (H1N1) (formerly H0N1) known for its amantadine resistance (Fig. 2). All other influenza A virus prototype strains tested previously by us (Heider *et al.*, 1980) proved to be sensitive.

Discussion

Information about strain-specific differences of influenza viruses regarding their inhibitor sensitivity is equivocal. While Schild and Sutton (1965) noticed a clear variability in the sensitivities of different strains to amantadine, other authors found only small variations among prototype strains and isolates from patients (Hayden *et al.*, 1980). These discrepancies are

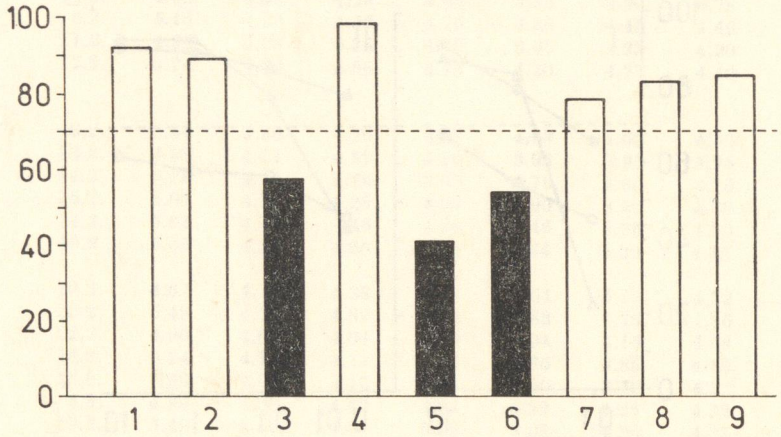

Fig. 2.

Sensitivity of different influenza A strains to rimantidine (1 μg/ml)
Area above the broken line: significant inhibition ($p < 5\%$).
Ordinate: inhibition %
Columns: 1 — A/Berlin/19/80 (H3N2)
2 — A/Berlin/36/80 (H3N2)
3 — A/Berlin/37/80 (H3N2)
4 — A/Krasnodar/101/59 (H2N2)
5 — resistant mutant of the latter
6 — A/PR/8/34 (H1N1)
7 — A/New Jersey/8/76 (Hsw1N1)
8 — A/PM/1/47 (H1N1)
9 — A/Hong Kong/8/68 (H3N2)

probably connected with the number of passages of the individual strains, since the sensitivity to amantadine declines with increasing passage number in animals (Tisdale and Bauer, 1975). The very frequently passaged H1N1 subtype virus A/PR/8/34, for example, displays a considerable amantadine resistance. But the passage history of the Berlin epidemic strains used in our study was quite uniform; they had undergone one to three chick embryo passages.

Although it is possible to isolate amantadine-resistant influenza A virus mutants under laboratory conditions in vitro, in ovo, or in vivo, attempts to isolate such mutants from treated patients have not been successful so far (Hayden *et al.*. 1980). The two inhibitor-resistant patient isolates from the 1980 epidemic (A/Berlin/21/80 and A/Berlin/37/80) confirmed Oxford's

(1974) prediction about the occurrence of resistant strains under natural conditions.

It is difficult to trace back the origin of our resistant strains since neither rimantadine nor amantadine are used at present in the G.D.R. and, therefore, resistant strains have no selective advantage in the population. These strains were possibly imported from countries where the drugs have been applied for several years (e.g. rimantadine in the U.S.S.R.). On the other hand, amantadine has been administered to patients with Parkinson disease for a long time, and so the selection of amantadine-resistant influenza viruses in this small population receiving long-term amantadine treatment is conceivable. The use of live vaccines with a PR/8 background could also enhance the drug resistance of "natural" influenza strains. The possibility that virus mutants selected for other properties reveal an altered inhibitor sensitivity (Presber *et al.*, 1974) should also be considered.

Whatever the reasons for the appearance of resistant strains, it is necessary to employ quick reliable methods of screening the influenza A epidemic strains for their reaction towards clinically used antiviral agents. For the purpose of screening, the haemadsorption reduction test (Adamczyk, 1977) appears superior to the plaque reduction test (Hayden *et al.*, 1980). The haemadsorption test is simple and cheap and results are obtained within 18 hr. Parallel studies in different Berlin laboratories have confirmed the reproducibility of the test. We are continuing these studies to assess the drug-resistance of current influenza A epidemic strains.

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