THE *IN VITRO* ACTIVITY OF 1-ADAMANTANAMINE HYDROCHLORIDE (AMANTADINE HCl) AGAINST SEVERAL STRAINS OF INFLUENZA VIRUS ISOLATED IN JAPAN

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Introduction

Since DAVIES and his colleagues first described the antiviral activity of 1-adamantanamine hydrochloride (amantadine HCl) against influenza virus[1], there have been many reports showing that this compound has an inhibitory effect on influenza A, A 1, A 2 and C and also on several members of myxovirus group when tested in cell culture system[2,3], in organ culture system[4], *in ovo*[3] or *in vivo*[2,5], In the following experiment, we examined the antiviral activity of amantadine HCl in monkey kidney cell culture system against several strains of influenza virus isolated in Japan. Antiviral activity was measured by means of the reduction in development of hemagglutinin by the viruses.

Materials and Methods

virus One strain of A1, 4 strains of A 2 and 1 strain of B influenza virus were tested in the experiment. All these strains, isolated in Japan during the epidemics for the past 10 years, were originally obtained from the National Institute of Health, Tokyo, and passed in primary monkey kidney(MK) cell or chorioallantoic membrane of the embryonated hen's egg several times in our laboratory(Table 1).

Table 1. Virus strains used in the experiment

virus strain*	passage history**	infectivity titer(EID_{50}/0.1 ml)
A 1/Omachi/1/53	E[1]-2	$10^{8.0}$
A 2/Adachi/2/57	E-30	$10^{7.8}$
A 2/Murakami/4/64	E-5	$10^{7.0}$
A 2/Kawasaki/1/65	E-9, MK[2]-1, E-7	$10^{7.5}$
A 2/Kumamoto/1/65	E-2, MK-1, E-1	$10^{7.5}$
B/Amakusa/1/64	E-3	$10^{5.6}$

* These virus strains were supplied by Dr. TAKEUCHI of NIH, Tokyo

** passage history in our laboratory (Original passage history is not clear.)

1) chorioallantoic membrane of embryonated hen's egg

2) monkey kidney cell

egg titration of the virus Serial tenfold dilution of the virus fluid was made with phosphate-buffered saline solution(PBS). 0.1 ml of each dilution was inoculated into allantoic sac of four 11-day-eggs. After 48 hours' incubation at 35°C, the eggs were chilled, the allantoic fluids were harvested and tested for the presence of hemagglutinin at the final dilution of 1 : 8 with 0.5% suspension of fowl red blood cell. 50% infectious dose (EID_{50}) was calculated according to the method of REED and MUENCH[6].

drug A sterile 10 w/v% stock solution of adamantanamine hydrochloride (amantadine HCl) was prepared in deionized water and stored at 4°C until required.

cell culture Primary cynomolgus or green monkey kidney cell was obtained from the commercial source. 3×10^5 cells were distributed per tube and grown in EARLE's balanced salt solution containing 0.5% lactalbumin hydrolysate and 2% bovine serum. When complete monolayer cell sheet was formed, experiment was started. Cell population at this stage was 75×10^4 per tube. Maintenance medium was PARKER's Medium 199 without serum and with 88 mg% $NaHCO_3$. All media contained 200 units of penicillin G and 200 mcg/ml of streptomycin.

toxicity of the drug to MK cell sheet Amantadine HCl was dissolved in various concentrations in Medium 199 and administered to MK cell sheet. 50

Table 2. Toxicity of amantadine HCl against MK cell sheet

conc. of amantadine HCl (mcg/ml) \ days of incubation	1	3	5	7
100	D	—	—	—
50	N	N	D	—
25	N	N	N	N
10	N	N	N	N
0	N	N	N	N

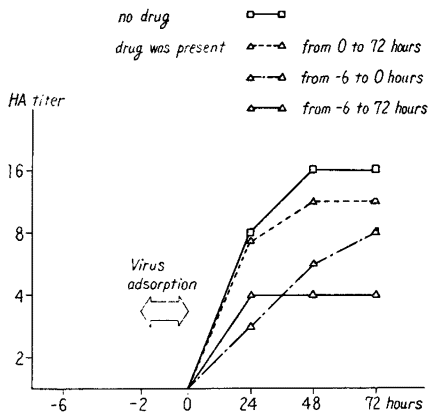
D=damaged N=not damaged

mcg/ml or higher concentration of the drug brought about gradual destruction of the cell sheet as observed under microscope, while the cell sheet remained intact throughout the incubation period if the concentration of the drug in the medium was 25 mcg/ml or less (Table 2). 25 mcg/ml was used in the experiment.

virus inoculation Cultures of MK cell were infected with a multiplicity of 10 to 80 EID_{50} per cell so that all the cells might be uniformly infected[7]. Cell sheet was washed three times with PBS, inoculated with 0.1 ml or virus fluid and incubated at 35°C for 90 minutes, after which unadsorbed virus was washed out with PBS, maintenance medium with or without the drug was added and incubation started.

drug administration 25 mcg/ml of amantadine HCl was administered to the drug-sensitive A 2/Kumamoto/1/65 strain of influenza virus in cynomolgus monkey kidney cell culture variously during the incubation period and the effect of the drug on free hemagglutinin production was estimated. As the greatest reduction in hemagglutinin production

Fig. 1 *Effect of amantadine HCl on free hemagglutinin production by A2/Kumamoto/1/65 strain of influenza virus in cell culture system, when the drug was administered variously during the incubation period.*



occurred when the compound was administered 4 hours prior to virus inoculation and was present during the entire period of incubation (Fig. 1), this mode of administration was adopted in the experiment.

hemagglutination test Serial two-fold dilution of culture fluid was made from each tube. To 0.4 ml of each dilution was added equal amount of 0.5% suspension of fowl red blood cell. After shaking, the tubes were let stand at room temperature and the pattern of agglutination was read at one hour.

Results

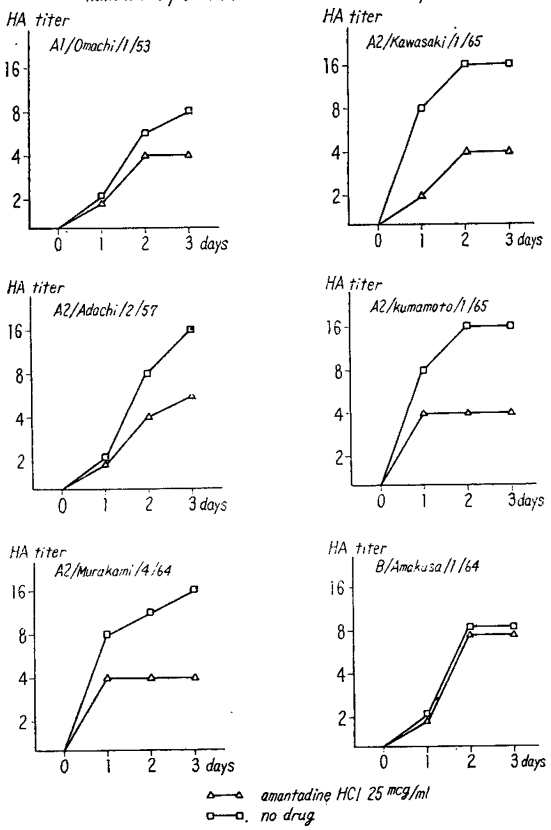
The results are schematically shown in Fig. 2 and summarized in Table 3. Murakami, Kawasaki and Kumamoto strain of influenza A 2 virus were strongly inhibited by 25 mcg/ml of amantadine HCl. Hemagglutinin developed by these viruses in the drug-treated groud remained 1 : 4 in its titer while the control showed as high as 1 : 16. A 2/Adachi/

Table 3. Summary of experiment

virus strain	cell culture system	input multiplicity (EID_{50}/cell)	incubation period (days)	dose of drug (mcg/ml)	reduction in HA titer
A 1/Omachi/1/53	GMK[1]	65	3	25	2
A 2/Adachi/2/57	CMK[2]	80	3	25	2.8
A 2/Murakami/4/64	CMK	10	3	25	4
A 2/Kawasaki/1/65	GMK	50	3	25	4
A 2/Kumamoto/1/65	CMK	50	3	25	4
B/Amakusa/1/64	GMK	20	3	25	—

1) green monkey kidney cell
2) cynomolgus monkey kidney cell

Fig. 2 *Effect of amantadine HCl on free hemagglutinin production by several strains of influenza virus in cell culture system. The drug was administered 4 hours prior to virus inoculation and was present for the whole of the incubation period.*



2/57 was less sensitive to the compound compared with these A 2 strains.

Only slight degree of inhibition was noticed in A 1/Omachi, and there was no inhibition in B/Amakusa strain of influenza virus.

Discussion

As has been reported by many investigators[8], inhibition by amantadine occurred predominantly in A 1 and A 2 strains of influenza virus, and influenza B was not affected by the compound at all.

It seems that pre-Asian strains of influenza virus is less sensitive to amantadine than A 2 strains. Thus A 1/Omachi strain, which was isolated in 1953, was inhibited less strongly than various strains of A 2 influenza viruses.

Even different strains of the same A 2 virus have different susceptibilities. Thus, SCHILD *et al.*[2] and NEUMAYER *et al.*[3] showed that there are different susceptibilities between A 2 influenza viruses such as Singapore/1/57, Japan/305/57, Scotland/49/57, AA/2/60, Tokyo/1/62 and 442/63 to the compound. In our experiment, there was no significant difference in susceptibility between Murakami/4/64, Kawasaki/1/65 and Kumamoto/1/65 strain of A 2 influenza virus, while A 2/Adachi/2/57 was less sensitive to amantadine compared with the other members of A 2 virus tested in the experiment. Whether this difference in susceptibility to amantadine is due to the difference in antigenic structure as is demonstrated by FUKUMI[9] is not clear.

There is a report indicating that antiviral activity of amantadine is due to inhibition of virus penetration into the host cell[10]. We could not obtain fully persuasive results concerning the mode of action of amantadine, although it has been shown that most striking inhibition was obtained when the compound was administered 4 hours prior to infection and was present fof the whole of the incubation period.

Summary

Antiviral activity against several strains of influenza virus isolated in Japan has been assessed in cell culture with 25 mcg/ml of amantadine HCl. All strains tested of A 2 influenza virus, *i. e.* A 2/Adachi/2/57, A 2/Murakami/4/64, A 2/Kawasaki/1/65 and A 2/Kumamoto/1/65, were strongly inhibited, while A 1/Omachi/1/53 was less sensitive and B/Amakusa/1/64 was completely resistant. Greatest activity occurred when the compound was administered 4 hours before virus inoculation and was present for the whole of the incubation period.

Acknowledgment

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