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Aminoadamantane-resistant strains of influenza A2 virus

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SUMMARY

After one passage of influenza A2/Singapore/1/57 virus in mice treated with 150 mg./kg./day of aminoadamantane, a partially drug-resistant strain of virus was detected in 1 of 12 mice. The isolation rate of aminoadamantane-resistant viruses increased to 8 after three passages in drug-treated mice. Some virus strains showed a 500-fold increase in resistance to aminoadamantane and to the structurally related compounds α-methyl-1-adamantane methylamine and 2-adamantanamine sulphate. No aminoadamantane-resistant viruses were detected after passage of influenza four times in mice treated with lower (15 or 1·5 mg./kg./day) concentrations of aminoadamantane. Aminoadamantane had no detectable effect on the development of lung lesions in mice infected with the drug-resistant influenza strain, whereas lung lesions were reduced in aminoadamantane treated mice infected with a control strain of influenza A2/Singapore virus. No differences were detected in the buoyant density in caesium chloride, morphology or serology between control and aminoadamantane-resistant strains of virus. These drug-resistant influenza viruses may be useful for detailed studies of the mode of action of aminoadamantane.

INTRODUCTION

1-Aminoadamantane hydrochloride has been shown to have a prophylactic and therapeutic effect (Galbraith, Oxford, Schild & Watson, 1970; Galbraith *et al.* 1971; Iezzoni, 1970) against influenza A2 infections in the general community. Acquired resistance to chemotherapeutic agents is an important problem in bacterial infections and studies have indicated that viruses can also acquire resistance to and dependence on antiviral compounds (Eggers & Tamm, 1961; Eggers & Tamm, 1963; Melnick, Crowther & Barrera-Oro, 1961; Renis & Buthala, 1965; Subak-Sharpe, Timbury & Williams, 1969). We have described previously the isolation of a strain of influenza A2 virus from infected mice being treated with aminoadamantane which showed a considerably increased resistance to the compound (Oxford, Logan & Potter, 1970).

Results are presented here showing the frequency of selection of such amino-adamantane-resistant strains and some of the biological characteristics of these

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drug-resistant influenza viruses. As well as the practical importance of the study of aminoadamantane-resistant influenza strains, such viruses would be useful for more detailed studies of the mode of action of aminoadamantane and this property of drug resistance may be useful as a marker for genetic studies with influenza viruses.

MATERIALS AND METHODS

Viruses

A mouse adapted strain of A2/Singapore/1/57 (H2N2) kindly supplied by Dr C. R. McDonald, Pfizer Ltd., Sandwich, Kent, was used. It had received four passages in eggs and 50 passages in mice. In our laboratory the virus received two further egg passages at limiting dilutions and was stored as an allantoic fluid pool at −80°C. For some experiments influenza virus was purified as described previously (Laver, 1969).

Chemicals

1-Aminoadamantane hydrochloride was kindly supplied by Dr A. Galbraith, Geigy (UK) Ltd., Macclesfield, Cheshire.

Quantitative haemadsorption (Q.H.) *test*

This test was used to measure the degree of inhibition of influenza viruses growing in tissue culture cells by aminoadamantane. The method described by Finter (1964) was followed with some modifications (Oxford, Potter & Logan, 1970). Aminoadamantane was diluted in twofold steps from 0·02 to 25 μg/ml. in mixture 199 and 1·5 ml. added per cell culture tube. After 15 min. incubation approximately 10 EID 50 of influenza virus per cell in 0·2 ml. was added to each tube. The cells were incubated for 19 hr. at 35–36°C. and then treated with a 0·5% suspension of guinea-pig red blood cells for 15 min. at 4°C. In control tissue culture tubes which were inoculated with virus only, the majority of the cells were infected and red blood cells haemadsorbed strongly to them. After two washings with phosphate-buffered saline pH 7·2 (PBS) at 4°C. to remove unadsorbed red blood cells, 1·5 ml. of deionized water at 37°C. was added per tube to lyse the haemadsorbed red blood cells. Eight tissue culture tubes of BSC–1 cells were used for each dilution of aminoadamantane and the relative haemoglobin concentrations were determined from O.D. readings at 410 nm. in a Unicam spectrophotometer. The optical densities were plotted against the concentration of aminoadamantane on a logarithmic scale, and the concentration of aminoadamantane inhibiting haemadsorption by 50% (inhibitory concentration: IC 50 in μg./ml.) was read off the resulting graph.

Production of drug-resistant strains by passage of influenza virus in aminoadamantane treated mice

The experimental protocol is summarized in Fig. 1. Adult Swiss white mice were inoculated intranasally under ether anaesthesia with 0·1 ml. of PBS, pH 7·2, containing 10^4 EID 50 of virus. Mice were killed after 72 hr., the lungs removed

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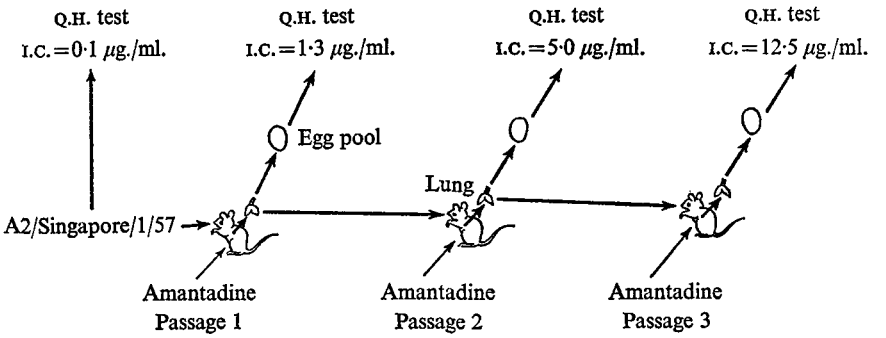

Fig. 1. Production of aminoadamantane- (Amantadine)-resistant strains of influenza virus.

aseptically and minced as a 10% (v/v) suspension in a mechanical blender in mixture 199. The suspensions were centrifuged at 2000 *g* for 10 min. and supernatant fluids frozen at −80°C. At each virus passage, mice were inoculated intraperitoneally with 50 mg./kg. of aminoadamantane 30 min. before inoculation with 10^4 EID50 of virus and supplied with drinking water containing 1 mg./ml. aminoadamantane. Separate tests established that mice received an approximate total of 150 mg./kg. aminoadamantane per day with this treatment.

Buoyant-density determination

One ml. of influenza haemagglutinin (diluted in 0·1 M tris HCl buffer, pH 7·4, to an HA titre of 1/128 approximately) was layered on the top of a preformed linear density gradient prepared from 2·4 ml. of 37% (w/v) and 2·1 ml. of 53% (w/v) CsCl dissolved in 0·1 M tris HCl buffer, pH 7·4. The tubes were centrifuged for 22 hr. at 100,000 *g* in the SW 39 rotor of a Spinco L preparative ultracentrifuge (Oxford & Potter, 1969).

RESULTS

The degree of inhibition of influenza viruses by aminoadamantane was estimated by quantitative haemadsorption (Q.H.). To determine the sensitivity and reproducibility of the Q.H. test two allantoic fluid pools of A2/Singapore/1/57 virus were tested for inhibition by the compound. A total of 13 Q.H. tests on different occasions using different batches of tissue culture cells with pool 1 gave a mean IC50 of 0·2 μg./ml. of aminoadamantane with a standard deviation of 0·19. Six Q.H. tests on influenza pool 2 gave a mean IC50 of 0·29 with a standard deviation of 0·10. The Q.H. test was thus a sensitive indicator of inhibition of virus growth and gave reproducible results.

Frequency of selection of aminoadamantane-resistant viruses

Influenza A2/Singapore/1/57 virus from a single egg pool was divided into 24 aliquots and these were used to infect 24 mice in individual cages. Twelve of these mice were treated with aminoadamantane (150 mg./kg./day) and the remaining 12 were control animals which received no drug. All mice were inoculated with

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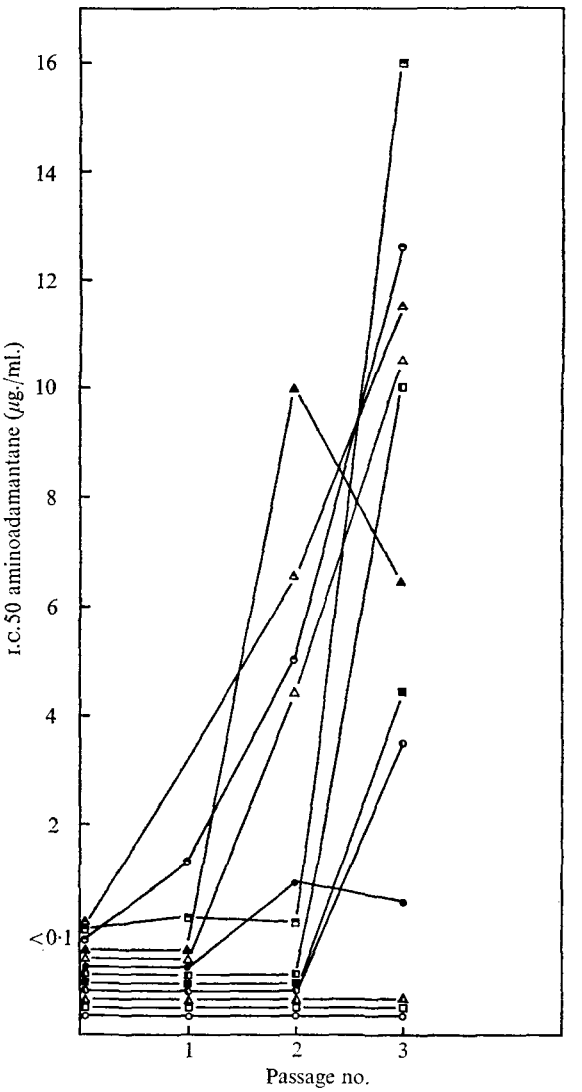

Fig. 2. Development of drug resistance during passage of influenza A2/Singapore/1/57 virus in aminoadmantane-treated mice.

approximately 10^4 EID 50 of virus intranasally. Lungs were harvested individually after 72 hr. and, after titration in eggs to determine the amount of infective virus, lung material was used to infect mice for the second passage (Fig. 1). After each of the three passages in mice, egg allantoic fluid pools were prepared from the infective lung material and the degree of inhibition of the isolated virus by aminoadamantane estimated by the Q.H. test.

The 36 virus pools from the control mice passage series were all inhibited by 0·02–0·2 μg./ml. of aminoadamantane in the Q.H. test. In contrast, influenza viruses resistant to inhibition by aminoadamantane were detected in a proportion of the 36 virus pools from drug-treated mice (Fig. 2). Drug-resistant viruses were

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Table 1. *Production of aminoadamantane-resistant viruses in mice treated with different concentrations of aminoadamantane*

Dosage of aminoadamantane (mg./kg./day)	Proportion of drug-resistant viruses isolated after 4 passes in mice
150	6/9
15	0/9
1·5	0/7

detected in the lungs of 1 of 12 mice on the first passage of virus in aminoadamantane treated animals. After three passages in drug-treated mice the proportion of aminoadamantane-resistant viruses increased and resistant viruses were present in eight of the twelve passage series. A single virus strain showed partial resistance to inhibition by aminoadamantane after one passage in mice: before passage in mice growth of the virus in tissue culture was inhibited by 0·1 μg./ml. of aminoadamantane and after one pass in drug-treated mice 1·3 μg./ml. aminoadamantane was required for inhibition (Fig. 1). After two and three passes in drug-treated mice 5 μg./ml. and 12·5 μg./ml. of aminoadamantane respectively were required for inhibition. Three virus strains required two passages in drug-treated mice before showing any resistance to inhibition by aminoadamantane, while in four strains drug resistance only became apparent after three passages in treated mice. Thus, strains of A2/Singapore/1/57 virus resistant to inhibition by aminoadamantane could be obtained with relatively high frequency after several passages of virus in mice treated with high concentrations of aminoadamantane.

In a second series of experiments influenza A2/Singapore/1/57 virus was passaged four times in groups of mice treated with varying concentrations of aminoadamantane (Table 1). Drug-resistant viruses were recovered only from mice treated with large doses (150 mg./kg./day) of aminoadamantane; no drug-resistant viruses were recovered from mice treated with 15 or 1·5 mg./kg./day of aminoadamantane.

Biological and physical characteristics of an aminoadamantane resistant virus strain

Virus was examined from a single pool which had been passaged three times in drug-treated mice and required 12·5 μg./ml. of aminoadamantane for inhibition in tissue culture compared to 0·02 μg./ml. of aminoadamantane before passage in mice. This resistant strain was also resistant to α-methyl-1-adamantane methylamine and 2-adamantanamine sulphate, antiviral compounds structurally related to 1-aminoadamantane. Resistance to aminoadamantane was a stable property of the virus since the strain remained drug-resistant after passage four times in eggs at limiting dilutions in the absence of aminoadamantane. The virus pool used for the following experiments was cloned by two passages at limiting dilution in eggs.

The buoyant density of the aminoadamantane-resistant and the control strain of influenza A2/Singapore/1/57 was determined in linear gradients of caesium chloride (Fig. 3). No significant differences were detected between the two viruses and the mean buoyant densities from three experiments were 1·24 g./cm.^{3} and

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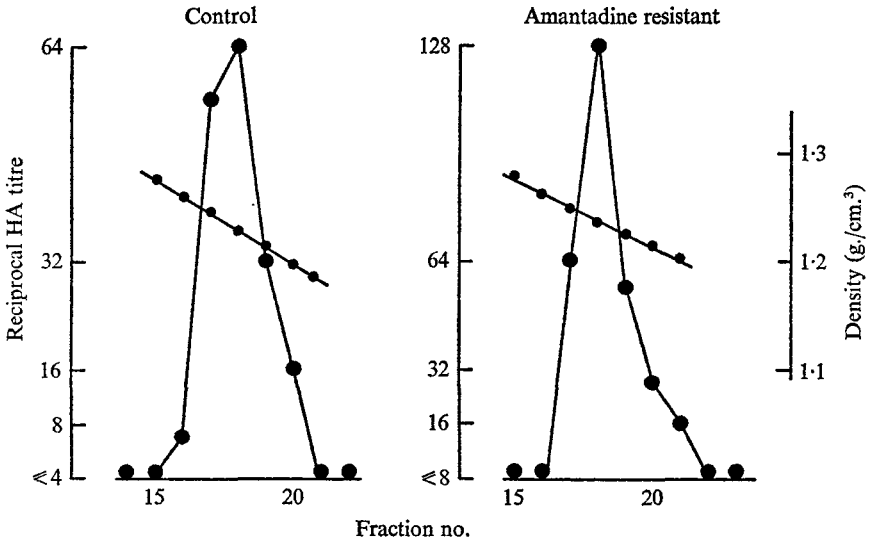

Fig. 3. Buoyant density in CsCl of aminoadamantane-resistant and control A2/Singapore/1/57 viruses. (A) Control virus. (B) Aminoadamantane-resistant virus.

1·23 g./cm.^{3} for the drug-resistant and the control strain respectively. Approximately 90% of virus haemagglutinin was recovered from the gradient with the control and aminoadamantane resistant strains. Electron microscopy (Oxford, Potter, McLaren & Hardy, 1971) showed preparations of the two strains purified by velocity-gradient centrifugation in sucrose to have a similar size and a non filamentous morphology typical of laboratory adapted strains of influenza virus.

No serological difference could be detected between the control strain and the drug-resistant strain. Haemagglutination and neuraminidase activities (Schild & Newman, 1969) of both viruses were inhibited to the same titre by a standard ferret antiserum* to influenza A2/Singapore/1/57 virus.

Finally, the aminoadamantane-resistant and the control virus were compared in *in vivo* studies to determine whether drug resistance was exhibited in animal experiments in addition to tissue culture (Table 2). The control strain of influenza A2/Singapore/1/57 caused lung lesions in mice 72 hr. after intranasal inoculation under ether anaesthesia and the amount of lung consolidation and the number of mice with lung lesions were both reduced significantly in mice treated with aminoadamantane. In contrast, aminoadamantane had no detectable effect on the incidence of lung lesions or on the degree of lung consolidation in mice infected with the aminoadamantane resistant strain of virus. Thus drug resistance was expressed in virus susceptible animals as well as in tissue culture.

Inhibition of influenza A2/Hong Kong/1/68 isolates by aminoadamantane

In an attempt to evaluate whether a proportion of naturally occurring influenza strains were resistant to inhibition by aminoadamantane, 30 freshly isolated

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Table 2. *Effect of aminoadamantane in mice infected with drug-resistant and control influenza* A2/*Singapore*/1/57 *viruses*

Virus	Treatment of mice	Lung lesion score (%) after infection with different virus dilutions:		
		−1	−2	−3
Aminoadamantane-sensitive	0	52*	40	32
	150 mg./kg./day aminoadamantane†	27	16	0
Aminoadamantane-resistant	0	92	82	28
	150 mg./kg./day aminoadamantane	86	89	31

* Expressed as percentage of maximum score. Ten mice per virus dilution. Lungs examined 72 hr. after infection.

† Fifty mg./kg. of aminoadamantane 30 min. before infection and 1 mg./ml. aminoadamantane in drinking water.

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Table 3. *Inhibition of influenza A2/Hong Kong/1/68 virus isolates by aminoadamantane*

Source of viruses	No. of viruses tested	IC50 conc. of aminoadamantane (μg./ml.) Range	Mean
Patients with influenza	26	0·02–0·26	0·09
Patients with influenza treated with 200 mg/day aminoadamantane	3	0·1–0·20	0·15
Contact with aminoadamantane-treated patient	1	0·08	–

strains of influenza A2/Hong Kong/1/68 virus were tested by quantitative haemadsorption (Table 3). All 30 strains were inhibited by 0·26 μg./ml. or less of aminoadamantane, including three strains which were recovered on the third day of illness from persons who had been treated with 200 mg. of aminoadamantane per day for 2 days, and one strain from a person in close familial contact with an aminoadamantane treated patient. Therefore no evidence of naturally occurring strains of influenza A2/Hong Kong/1/68 resistant to aminoadamantane was detected in this preliminary study.

DISCUSSION

The results indicate that drug-resistant strains of influenza A2/Singapore/1/57 virus can be selected with relatively high frequency after three passages in mice treated with high concentrations of aminoadamantane. The degree of drug resistance detected was considerable, particularly in viruses recovered after three passages in aminoadamantane-treated animals: some strains showed a 500-fold increase in resistance to the compound. However, no direct conclusions can be drawn from the present study about the possibility of emergence of aminoadamantane-resistant influenza strains in humans. The mice used in the present study were treated with relatively large concentrations of aminoadamantane (150 mg./kg./day) compared to the dosage in humans of 200 mg. per person per day. No drug-resistant strains were detected during passage of virus in mice treated with 15 or 1·5 mg./kg./day of aminoadamantane. Only three virus strains isolated from persons being treated with aminoadamantane were available for testing, and although these were all very sensitive to inhibition by aminoadamantane, further studies with many more strains are required.

Particularly interesting was the failure to detect differences in biological and physical properties between control influenza strains and aminoadamantane-resistant strains. Thus, the surface antigens of the two strains were similar. In addition, the buoyant densities in caesium chloride of the two virus strains were not significantly different. Aminoadamantane acts at an early stage of virus multiplication and may prevent virus penetration (Hoffmann, Neumayer, Haff & Goldsby, 1965) or uncoating (Kato & Eggers, 1969; Long & Olusanya, 1972) of the virus genome following the penetration step. Preliminary investigations have

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failed to detect any effect of aminoadamantane on the activity of influenza-virion-associated RNA-dependent RNA polymerase (J. S. Oxford, in preparation). It might be expected that differences between the normal and drug-resistant viruses would be detected at the point of action of aminoadamantane; thus differences may occur in the method or rate of penetration or uncoating between the viruses. The use of aminoadamantane-resistant strains of influenza may help to elucidate the mode of action of the compound, and may lead to a more exact definition of the early stages of influenza infection in the cell.

The relatively mild antiviral activity of even large doses of aminoadamantane in mice and the break-through of influenza virus in treated tissue cultures (Oxford & Schild, 1968) noted in previous studies would seem to indicate a heterogeneous population of influenza virions containing a proportion of resistant particles. However, it is not possible to conclude from the results of the present study whether drug-resistant strains emerged by mutation and selection or more simply by selection from a heterogeneous virus population. Studies in tissue culture using plaque-purified viruses and more defined conditions of growth are required to answer these questions. Studies in humans have indicated that subclinical and clinical influenza may still occur in a proportion of persons receiving amino-adamantane prophylactically and that virus may be recovered from throat swabs in titres comparable to those from normally infected persons not receiving aminoadamantane (Iezzoni, 1970). Therefore it might be possible for any amino-adamantane-resistant virions selected in a person receiving the compound prophylactically or therapeutically to be subsequently transmitted. It is suggested that a surveillance of any possible emergence of drug-resistant influenza strains should be kept in future trials of aminoadamantane and other anti-influenza compounds.

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