STUDIES ON THE ANTIVIRAL ACTIVITY OF 1-ADAMANTANAMINE

D. A. J. TYRRELL, M. L. BYNOE AND B. HOORN

From the M.R.C. Common Cold Research Unit, Salisbury, England and the Department of Bacteriology of The University of Lund, Sweden

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THERE are many substances both natural and synthetic which inhibit the multiplication of influenza viruses. Many of these are also very toxic for cells and others are difficult to manufacture so that relatively few can be considered as possible antiviral chemotherapeutic agents. One such possible substance is 1-adamantanamine which is very active *in vitro* and has been shown to slow down virus multiplication and the development of lung lesions when administered to mice infected with influenza A virus (Davies *et al.*, 1964).

As 1-adamantanamine has antiviral activity against only certain viruses in each biological group, we performed preliminary tests *in vitro* to determine whether it would inhibit multiplication of certain of our strains of viruses in human cells ; we then attempted to prevent infection and illness following the administration to volunteers of a strain of attenuated influenza A2 virus which was very sensitive to the drug in tissue cultures. The results are summarized in this paper.

MATERIALS AND METHODS

Tissue cultures

Primary trypsinized cells.—Human embryo kidney and monkey kidney cells were trypsinized and grown in roller tubes by standard techniques. When influenza virus was to be used all cultures were washed 3 times and fed with 1 ml. of medium 199 before inoculation.

Diploid human embryo fibroblast cells.—A strain isolated in this laboratory was used and maintained in medium 199 for experiments with influenza virus. Media containing 2 per cent calf serum and with a pH about 7·2–7·4 were used for experiments on rhinoviruses (Parsons and Tyrrell, 1961).

Organ cultures were prepared from human embryo trachea and nose and maintained in medium 199 as described by Hoorn (1964).

Virus diagnostic tests

Nasal washings were collected from all volunteers on the second, third and fourth day after inoculation, and sera were collected before inoculation and about 2 weeks later. The washings were collected in buffered saline, mixed with an equal volume of broth and stored at −70° or tested immediately by inoculation into the allantoic cavity of 10 to 11-day-old eggs. The sera were tested by haemagglutination-inhibition against the A2/Pak/1/57 virus using human group O red cells and performing the tests by the micromethod of Takátsy (Takátsy and Furesz, 1954). When possible sera were also tested by a new quantitative neutralization test using the IKSHA strain of influenza A2 (Finter, unpublished). The tests were performed by Dr. N. Finter, I.C.I. Laboratories, Alderley Edge, Cheshire.

Virus strains

Most of these have been described elsewhere. A2/Pakistan/1/57 by Himmelweit (1960), the vaccine strains IKSHA, A2/Scot/49/57 and A2/Eng/43/57 by Zuckermann (personal communication) and the rhinoviruses, H.G.P., P.K. and J.H. by Tyrrell and Bynoe (1961) and Price (1956). The vaccine strains were part of the pools prepared for large-scale tests on human volunteers. The organ cultures were infected with an influenza A2 strain isolated in Copenhagen in 1957 and passed 20 times in eggs and 43 times in organ cultures of human respiratory epithelium.

RESULTS

Experiments in tissue culture

Preliminary experiments showed that multiplication of influenza A2 virus was inhibited by 30 μg./ml. of adamantanamine, but one recent strain of influenza B was unaffected. Table I shows that there is a powerful effect on the multiplication

TABLE I.—*Inhibitory Activity of Adamantanamine in Roller Tube Cultures of Trypsin Dispersed Human Embryo Kidney and Monkey Kidney Cells Inoculated with 8HA Units of A2/Pak/1/57*

Type of celll	Addition of adamantanamine.	Haemagglutinin titre of medium	Fraction of cell sheet showing haemadsorption per cent
Human embryo kidney	No	64, 32, 64, 64	20, 50, 20, 50
	Yes	1, 0, 0	0, 1
Monkey kidney	No	4, 4, 4, 4	90, 90, 70, 80
	Yes	0, 0, 0, 0	0, 10, 5, 1
Human embryo lung fibroblasts	No	1, 0, 0, 0	10, 10, 20
	Yes	0, 0, 0, 0	0, 0, 0

* The medium contained the drug at a concentration of 30 μg./ml. from the time of inoculation and was renewed and titrated daily. The results shown are those for 3 days after inoculation.

of influenza A2 in cultures of human embryo kidney and monkey kidney cells. Treated cells released little haemagglutinating virus into the medium and, as judged by haemadsorption, few of them had virus at the cell surface either. These results were confirmed in several experiments using reduced doses of virus, about 0·08 HA units per culture. We wished to use rhinoviruses for experiments on human volunteers, but as is shown in Table II there was no evidence of activity against several M rhinoviruses.

TABLE II.—*Lack of Inhibitory Activity of Adamantanamine* (30 μg./ml.) *in Roller Tube Cultures Infected with M Rhinoviruses*

Virus	Cells	Total number of microplaques in tubes containing: adamantanamine	Total number of microplaques in tubes containing: no drug
ECHO 28	Monkey kidney	17	16
H.G.P.	Monkey kidney	42	35
H.G.P.	Human kidney	93	60
P.K.	Human embryo fibroblasts	66	58

We also wished to know if the drug would inhibit the growth and cytopathic effect of the virus in human ciliated respiratory epithelium in organ cultures. It is known that foetal human trachea in organ culture reacts to influenza A2 virus infection in a similar manner to that observed *in vivo* (Hoorn, 1964). Organ

cultures were therefore prepared and maintained in medium 199 containing 30 μg./ml. adamantanamine. These were infected with influenza A2 virus and incubated at 33° ; the medium was changed daily and the virus concentration measured by titration *in ovo*. The ciliary activity was observed by microscopy, and at the end of the experiment the tissue fragments were fixed and sectioned. A typical growth curve and section are shown in Fig. 1 and 2 respectively. These

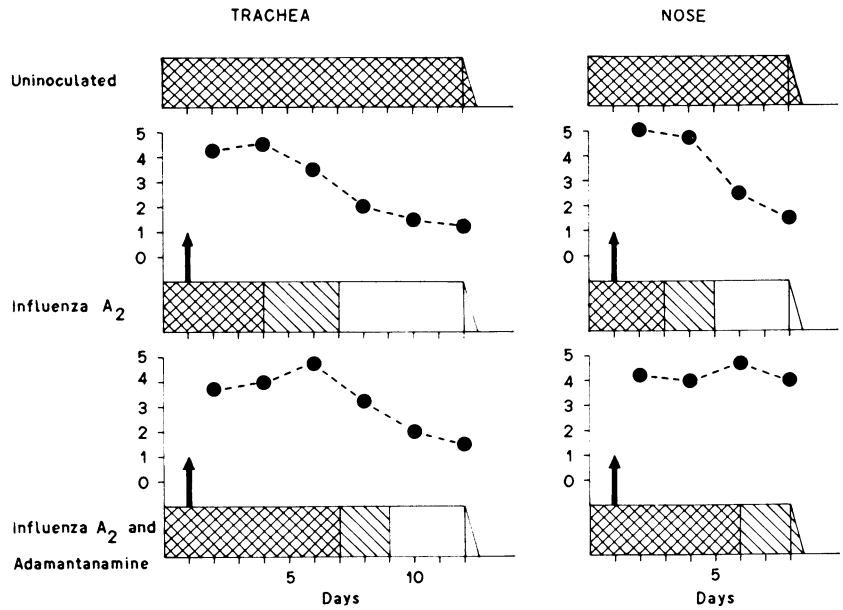
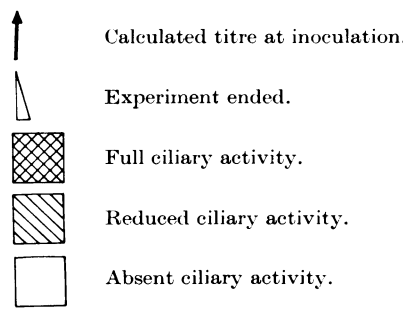
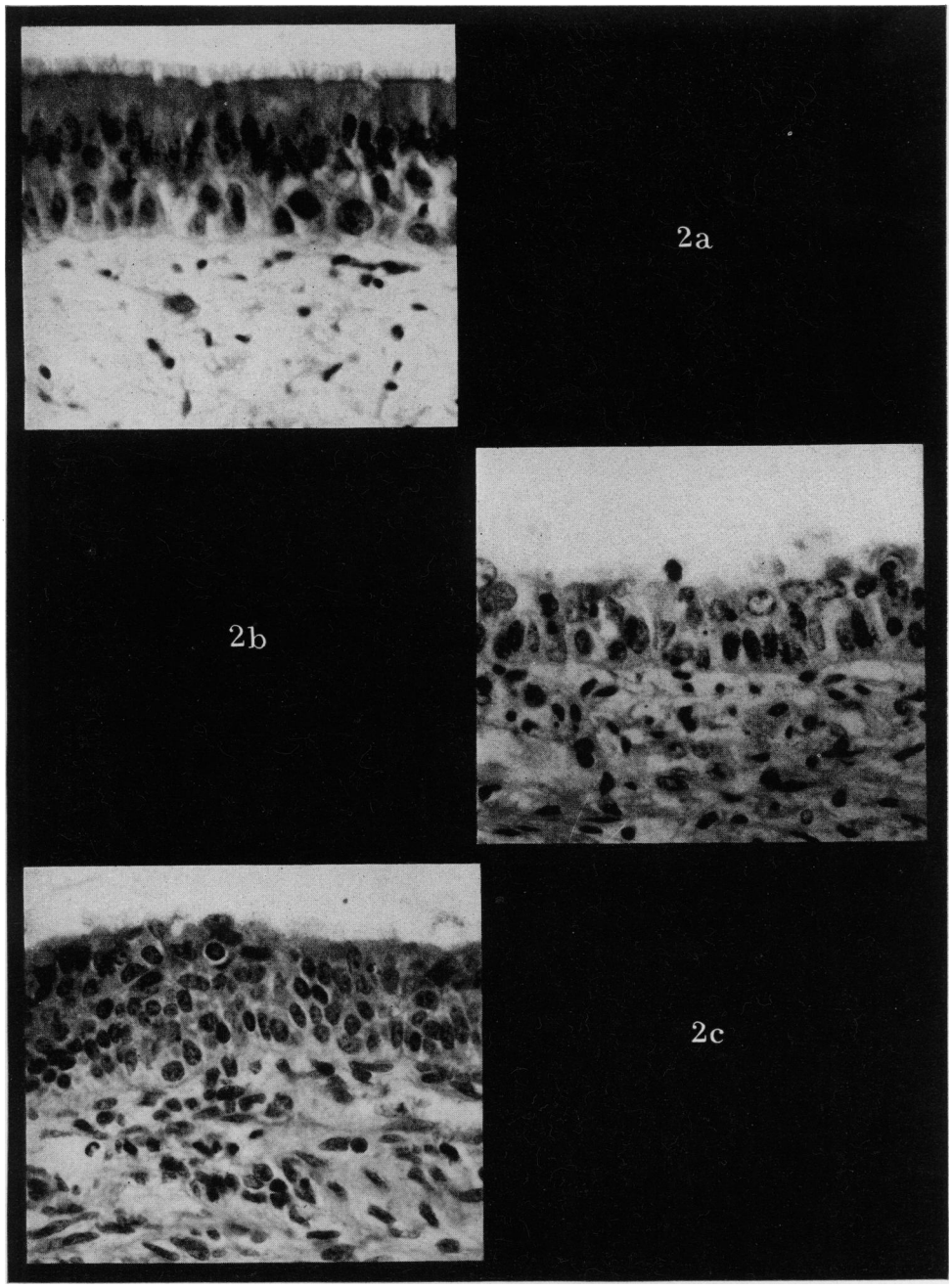


FIG. 1.—The effect of the presence of adamantanamine (30 μg./ml.) in the medium of organ cultures infected with influenza A2. As shown by the horizontal bars, the ciliary activity was present throughout the experiment in the control cultures ; it was reduced or abolished later in treated than in untreated cultures. Egg infectivity titres of the culture fluids are shown above the corresponding bar.



EXPLANATION OF PLATE

FIG. 2.—Fixed and stained epithelium from organ cultures of human embryo nasal epithelium. 2*a* The culture maintained for 7 days. 2*b* The culture inoculated with influenza A2, and 2*c* the culture which was infected with the virus but maintained with a medium containing 30μg./ml. of adamantanamine. H. and E. (×500 approx).



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show that destruction of cilia by the virus was delayed by the drug, which also slowed down the first wave of multiplication of the virus ; later on, more virus was produced by the treated than by the untreated cultures, presumably because the epithelium of the former was less damaged than that of the latter. This experiment showed that the multiplication of the virus and its harmful effect on the cells were somewhat delayed by the drug. The drug was also shown to be harmless for these organ cultures.

As the drug seemed to be very active in cultures of trypsin dispersed human cells and to some extent in human organ cultures, we performed experiments in man with the main object of showing whether it had any effect on the clinical manifestations of an experimental infection with influenza A2 virus.

Experiments on volunteers

Volunteers were kept in isolation and in the first tests received 2 capsules of 200 mg. of drug night and morning from 2 days before inoculation to 4 days after. Volunteers receiving no drug received identical-appearing capsules containing lactose. The virus was given as 1 ml. of nasal drops prepared by diluting allantoic fluids 1/10 or 1/100 in chilled Hanks' saline just before inoculation.

The experiments were conducted step by step in order to avoid toxic effects. As may be seen in Table III two volunteers in the first experiment showed trouble-

TABLE III.—*Summary of Experiments in Volunteers*

Trial No.	Virus strain given	Dose EID_{50}	Dose of drug mg./day	Proportion of volunteers showing: Illness	Proportion of volunteers showing: Laboratory evidence of infection
16	A2/Eng/443/57	?	400	*1/4	1/4
			0	1/3	1/3
	None		400	*1/3	—
			0	0/3	—
17	A2/Scot/49/57	10^6	400	0/3	2/3
	,,	,,	0	1/3	1/3
18	A2/Scot/49/57	10^7	400	3/4	4/4
	,,	,,	0	1/4	3/4
19	,,	,,	200	1/3	2/3
	,,	,,	0	1/3	1/3
17 to 19	A2/Scot49/57	10^6 or 10^7	200 or 400	4/10	8/10
combined		10^6 or 10^7	0	3/10	5/10

* Malaise, tremors and insomnia—other subjects developed upper respiratory tract symptoms but some of those given drug had insomnia from time to time.

some toxic effects, on the 2nd and 3rd days after administration of the drug ; the symptoms and signs disappeared rapidly when the drug was discontinued. As the virus had little effect we changed to another strain which we expected to produce more antibody responses, and maintained the high dosage of the drug in the hope of showing a clear-cut effect on the virus infection. After trial 18, insomnia had been so troublesome among the volunteers that the dose was reduced to 200 mg. once daily. Table III shows that 4 of 13 volunteers given virus alone developed respiratory illness while 4 of 14 given the drug as well as a virus did so. Similarly, 6

TABLE IV.—*Summary of Results of Laboratory Tests*

Virus given	Drug given	Proportion of volunteers showing: Virus in throat	Rising antibody titre by: H.I.	Rising antibody titre by: Neutralization*
A2/Eng/443/57	Yes	1/4	0/3	1/3
	No	1/3	0/3	0/2
A2/Scot/49/57	Yes	2/10	6/10	3/8
	No	1/10	4/10	5/6

* Not all sera were tested.

of 13 given virus and placebo showed laboratory evidence of infection while 9 of 14 given virus and drug did so. Seven of the 9 volunteers who developed a respiratory illness were shown to be infected by laboratory tests. Table IV shows that the frequency of virus isolations and of antibody rises as detected by both techniques did not differ significantly in the treated and untreated groups. Ten of the volunteers given A2 Scot/49/57 virus had antibody in the pre-inoculation serum which could be detected by H.I. tests. It was concluded that there was no evidence that the drug prevented infection of volunteers with the virus used or the signs and symptoms of disease.

DISCUSSION

Our results confirm that adamantanamine is, as its discoverers claim (Davies *et al.*, 1964), a relatively non-toxic substance with a powerful inhibitory activity against influenza A2 *in vitro*. Although virus strains vary greatly in their sensitivity to the drug (Schild and Sutton, 1965), the strain used in our experiments on volunteers is very susceptible to its action. Furthermore, since inhibitory activity was detected in several different types of tissue culture and to some extent in organ cultures of human respiratory epithelium the inhibitory effect could be expected in man.

As it was not found, we wondered whether the virus infection produced was materially different from that *in vitro*. Large doses of virus were used both *in vitro* and in volunteers. Nevertheless, if we had infected volunteers who had no antibody with a small dose of a virus which then passed through many cycles, we might have had a better chance to detect activity if it were weaker than in tissue cultures. Egg-adapted viruses will not infect man if given in small doses. We could not select volunteers without antibody and we did not regard it as ethical to give an unattenuated virus to volunteers. We were therefore unable to devise an improved way of doing tests in volunteers. Nevertheless, if there had been a marked effect on the virus infection this would have been detected by one or other of the tests used.

Judging from data supplied by the manufacturers it is likely that the concentration of the drug in the respiratory epithelium was significantly lower than that in the tissue culture fluids, and this we feel was a further factor behind the lack of effects which we observed. We feel that in future studies of the antiviral effect of drugs in respiratory virus diseases an attempt should be made to study their action in an experimental set-up which resembles the natural infection more closely than the influenza virus infection of tissue cultures and mice which were used in this case. Organ cultures might be very useful for this purpose, but in our experiments

we could not decide whether the rather limited degree of protection observed was due to the cell type or to the strain of virus used.

It is still possible that the drug is active when human beings without antibody are infected with a wild influenza virus as in natural infections and recently it was reported that there was serological evidence that volunteers without antibody were partly protected from infection by the drug (Jackson, Muldoon and Akers, 1964). Further experiments are needed not only to study the practical value of adamantanamine, but also to define better the optimal method of selecting and studying antiviral drugs in experiments on volunteers.

SUMMARY

1-Adamantanamine inhibited the multiplication of certain strains of influenza A2 virus in trypsin-dispersed cultures of human and monkey kidney cells, and somewhat delayed the effects of the virus on organ cultures of human tracheal epithelium. The drug had no effect on the growth of the strains of influenza B or rhinoviruses used. No clinical or laboratory evidence for an antiviral effect was observed in a double-blind trial on 33 volunteers who lived in isolation and were infected with influenza A2 virus.

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