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Laboratory characteristics of an attenuated influenza type A (H3N2) virus ('Alice' strain)

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SUMMARY

The Alice strain of live attenuated influenza virus was obtained by selection of a γ inhibitor-resistant strain from a virus recombinant between A/PR/8/34 (H0N1) and A/England/42/72 (H3N2). Its behaviour *in vitro* and *in vivo* was studied. Three marker systems were investigated: resistance to serum inhibitors, growth capacity at high temperature and low sensitivity to amantadine hydrochloride. In ferrets the strain was found to be attenuated and immunogenic. Passages in man, animals and eggs have not affected its resistance to γ inhibitors.

INTRODUCTION

The various methods used for the attenuation of influenza virus were reviewed recently by Kilbourne *et al.* (1974). Selection of variant viruses resistant to γ type non-specific serum inhibitors has been reported to result in attenuation for Asian (H2N2) and Hong Kong (H3N2) subtypes (Soloviev & Neklyudova, 1969; Beare & Bynoe, 1969; Lamy *et al.* 1973). More recently, it has been shown that genetic recombination between attenuated and virulent influenza type A viruses resulted frequently in reduction of virulence for man and animals (Beare & Hall, 1971; Murphy, Chalhub, Nusinoff & Chanock, 1972; Maassab, Kendal & Davenport, 1972; Hara, Beare & Tyrrell, 1974).

In the present paper we describe the laboratory characteristics of an attenuated virus ('Alice' strain) obtained by a combination of these two methods.

MATERIAL AND METHODS

Viruses

The Alice strain is an inhibitor-resistant variant of the MRC-2 strain. It was obtained by passages of the MRC-2 strain in the presence of normal heated guinea-pig serum and cloned by three further passages, using endpoint dilutions in the absence of serum.

Strains MRC-2 (a recombinant between A/PR/8/34 and A/England/42/72 with the antigenic composition of the latter), A/England/42/72 (H3N2), A/Victoria/101/

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72 (H3N2) and A/PR/8/34 (H0N1) were received from Dr Schild, World Influenza Centre, National Institute for Medical Research, Mill Hill, London.

Reisolates of the Alice strain were obtained from nasal washings of volunteers on days 1 and 2 after inoculation; in one instance the reisolate came from a sample of the fourth day post-inoculation.

Eggs

Fertile eggs were purchased from an SPF flock (Lohmann Tierzucht GmbH, 219 Cuxhaven, Postfach 460, Germany); eggs used for titrations were of conventional origin.

Non-specific inhibitors

Guinea-pig and horse sera were diluted 1/2 in phosphate buffered saline (PBS), pH 7·4, and heated at 75° C. for 10 minutes. They were then centrifuged for 30 min. at 2000 rev./min. The supernatant was used as a source of γ inhibitors in hemagglutination-inhibition and infectivity titrations.

Hemagglutination-inhibition tests (HI)

For antibody determinations, sera were inactivated at 56° C., for 30 min. and treated with a 25% suspension of kaolin (Spence, 1960). Four to eight haemagglutinating units were mixed with twofold serum dilutions and left in contact for one hour at room temperature. A 0·5% suspension of chicken erythrocytes was then added. The HI titres were expressed as reciprocals of the dilution at which the haemagglutination was completely inhibited.

Infectivity titrations

Infectivity titrations were done in 9- to 11-day-old fertile eggs using 0·2 ml. per egg via the allantoic route. The titres were expressed as egg infectious doses 50% (EID 50). For comparative titrations in the presence or absence of serum γ inhibitors, tenfold virus dilutions were made in PBS. One volume of each dilution was added to one volume of a 1/2 dilution of heated guinea-pig serum or to one volume of PBS. The mixtures were kept for 1 hr. at room temperature and then inoculated by the allantoic route into 4–5 fertile eggs per dilution. The eggs were tested for the presence of haemagglutinin after an incubation period of 72 hr. at 35° C. In comparative infectivity titrations at 35 and 39·5° C., tenfold virus dilutions in PBS were inoculated into 8–10 eggs per dilution; 4–5 eggs of each dilution were incubated at each temperature for a period of 72 hr. before being tested for the presence of viral haemagglutinin.

Chicken kidney tissue cultures (CKTC)

Cultures were made according to the method described by Beare & Keast (1974). Eagle's basal medium (BME) was used as maintenance medium.

Sensitivity to amantadine hydrochloride

Comparative infectivity titrations in CKTC were carried out in the presence and absence of amantadine hydrochloride (Endo Laboratories Inc.). Tubes were pretreated for three hours at room temperature with BME containing 25 μg./ml. of

the drug. At the end of this period, four treated and four untreated tubes were inoculated with 0·1 ml. of the same serial tenfold virus dilution. After 5–7 days incubation at 35° C., the supernatants were tested for the presence of viral haemagglutinin. The last positive dilution was considered as the infectious titre.

Amantadine sensitivity was expressed as the difference in infectious titre obtained in the presence and absence of amantadine.

Experiments in animals

Experiments in hamsters. Inocula with titres ranging between 10^3 and 10^7 EID 50 of the Alice strain were administered intranasally to groups of 4 hamsters per dilution. Two control hamsters were housed with the group which had received 10^7 EID 50. All animals were examined daily. Nasal washings were made on days 1, 2, 4 and 7 in the groups which had received 10^3, 10^5 or 10^7 EID 50 and were tested for virus content in eggs. Virus positive allantoic fluids were checked for their serotype and resistance to serum inhibitors. The animals were bled 15 days after inoculation and serum samples were tested for the presence of HI antibody.

Experiments in ferrets. In comparative trials, groups of ferrets were inoculated with the Alice strain or with wild type strains. Body temperature, viral excretion and immunological responses after virus administration were evaluated following methods and criteria described by Potter *et al.* (1972) with the following modification: sera for HI antibody determination were treated with kaolin as described above.

RESULTS

Resistance to serum inhibitors

Strains A/England/42/72 and MRC-2 and samples of 3 different preparations of the 'Alice' strain (the seed lot and experimental lots 1 and 2) were tested in HI tests using horse and guinea-pig sera and in comparative infectivity titrations in the presence and absence of guinea-pig serum. Also, the stability of the inhibitor-resistant property was tested by checking the tenth serial egg passage of undiluted Alice strain and also of virus reisolates from human vaccinees and animals. As can be seen in Table 1, the haemagglutination of the parent strain was strongly inhibited by guinea-pig and horse sera. Also, the infectivity titre of the MRC-2 strain was reduced by 10^6 EID 50 in the presence of guinea-pig serum. In contrast, the different preparations of the Alice strain tested were insensitive to guinea-pig or horse serum inhibitors. All the reisolates from inoculated animals or from human subjects were resistant to guinea-pig serum.

Growth at high temperature

Comparative infectivity titrations were done in eggs incubated at 35 or 39·5° C. using the parent and vaccine strains as well as some of the virus reisolates from human vaccinees. The results are shown in Table 2. For the Alice strain approximately the same titre was obtained at both temperatures whereas the parent strain failed to grow at 39·5° C. The virus samples recovered from vaccinees behaved like the vaccine virus.

Table 1. *Sensitivity of the Alice strain and parent strains to serum inhibitors*

Virus	HI titre		Infective titre* in presence of	
	Horse serum	Guinea-pig serum	PBS	Guinea-pig serum
Parent strains				
A/England/42/72	51,200	12,800	NT	NT
MRC-2 recombinant	51,200	25,600	6·8	0·75
Alice strain				
Seed lot	< 8	< 8	6·5	6·5
Lot 1	< 8	< 8	6·0	6·0
Lot 2	< 8	< 8	6·3	6·3
5th egg passage	NT	< 8	NT	NT
10th egg passage	NT	< 8	4·8	5·5
Alice reisolates				
from humans	NT	< 8 (13/13)†	NT	NT
from ferrets	NT	< 8 (24/24)†	NT	NT
from hamsters	NT	< 8 (29/29)†	NT	NT

* Expressed as $\log_{10}$ EID 50/0·2 ml.
† No. of reisolates < 8/no. of reisolates tested.

Table 2. *Growth capacity of Alice strain and parent strains at* 39·5° C.

	$\log_{10}$ EID 50/0·2 ml. at	
	35° C.	39·5° C.
A/England/42/72	5·3	⩽ 0·5
MRC-2	7·3	⩽ 0·5
Alice	6·5	6·0
Reisolates from humans: no. 221·2	6·0	5·7
247·1	6·5	5·75

Amantadine sensitivity

Several comparative CKTC infectivity titrations were carried out in the presence and absence of amantadine hydrochloride. A/PR/8/34 (H0N1), A/England/42/72 (H3N2), MRC-2 (H3N2) and Alice strains were compared. A/England/42/72 strain was consistently inhibited by at least 3 log whereas the other strains were never inhibited by more than 2 log and usually by 1 log.

Experiments in hamsters

Groups of 4 hamsters each were inoculated with tenfold dilutions of the Alice strain as shown in Table 3. Pathogenicity, virus excretion, spreading potential and immunogenicity were studied. The results demonstrated that the virus was excreted during at least 7 days by some animals but the peak seemed to be in the first 4 days. The virus spread to one of the two hamsters in contact with those which had received the highest virus inoculum. The HI titres reached after infection were not influenced by the titre of the virus inoculum provided this dose was sufficient to infect the animals. As determined by serologic response, the minimal hamster infective dose was found to be approximately 10^3 EID 50.

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Table 3. *Dose range in hamsters*

Cage no.	Titre of inoculum (EID 50)	Number of animals: In test	Excreting virus on (days after inoculation): 1	2	4	7	Total	With serum HI titres against A/England/42/72 of: < 8	32	128	256	512
1	0 (control)	2	0	0	1	1	1/2	1	0	1	0	0
	10^7	4	3	4	2	0	4/4	0	0	2	1	0
2	10^6	4	NT	NT	NT	NT		0	0	2	1	0
3	10^5	4	3	3	4	1	4/4	0	0	1	2	1
4	10^4	4	NT	NT	NT	NT		0	0	2	1	0
5	10^3	4	0	1	3	1	3/4	1	0	2	1	0
6	10^2	4	NT	NT	NT	NT		4	0	0	0	0

NT = Not tested.

Table 4. *Response of ferrets to intranasal inoculation with the Alice strain and and with homologous wild type viruses*

Ferret no.	Virus strain	Inoculum (EID 50) ferret	Body temp. ⩾ 40° C.	⩾ 1° C.	Virus isolation	HI antibody titre: 0	3 weeks
108	Alice	$10^{6\cdot5}$	−	−	+	< 16	1024
109	Alice	$10^{6\cdot5}$	−	−	+	< 16	1024
64	Alice	$10^{7\cdot4}$	−	−	+	< 8	2048
66	Alice	$10^{7\cdot4}$	−	−	+	< 8	⩾ 512
277	Alice	$10^{7\cdot4}$	−	−	+	< 8	⩾ 4096
114	A/England/42/72	$10^{6\cdot0}$	−	+	NT	< 16	1024
115	A/England/42/72	$10^{6\cdot0}$	−	+	NT	< 16	512
112	A/Victoria/101/72	$10^{7\cdot5}$	+	+	NT	< 16	256
107	A/Victoria/101/72	$10^{7\cdot5}$	+	+	NT	< 16	1024

Virus recovered from eggs inoculated with nasal washings and lung homogenates was found to be γ type inhibitor resistant (Table 1).

Experiments in ferrets

The results obtained after a primary inoculation of ferrets with Alice and wild homologous strains A/England/42/72 and A/Victoria/101/72 are recorded in Table 4. The ferrets inoculated with the Alice strain did not show any significant increase in body temperature based upon the criteria defined by Potter *et al.* (1972) (two readings ⩾ 40° C. and two readings ⩾ 1·0° C. above the mean pre-inoculation temperature occurring 24–72 hr. after inoculation). In contrast, the animals inoculated with the wild strains A/England/42/72 and A/Victoria/101/72 showed hyperthermia. Virus shedding was measured for four consecutive days after immunization with Alice. The virus isolates were found to be resistant to γ serum inhibitors present in guinea-pig sera (Table 1). The homologous HI titres three weeks after inoculation with Alice or wild type viruses were ⩾ 256 for all the ferrets.

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DISCUSSION

The results obtained *in vitro* and *in vivo* demonstrated that the Alice strain has genetic markers which are useful in the identification of the virus. The strain has been defined as a γ type inhibitor resistant strain because its haemagglutination and infectivity are not impaired by heated guinea-pig and/or horse sera which contain this inhibitor at high titres (Shortridge & Landsell, 1972). The γ type inhibitor resistance of the Alice strain was shown to be stable after passage in human volunteers, ferrets and hamsters. This observation corroborates the results of Rubin *et al.* (1974) who tested virus reisolated from human volunteers vaccinated with Alice and found it to be identical with the vaccine strain. This finding is also in accordance with our previous results with another inhibitor-resistant strain, the Ann strain. This strain was also shown to maintain its inhibitor-resistance after passage in man, animals or eggs (Huygelen *et al.* 1973).

There is no sensitive method for checking the presence of a few inhibitor sensitive particles in a resistant viral population but, according to literature reports the growth of sensitive particles seems to be favoured by passages in eggs (Choppin & Tamm, 1960; Barb & Takatsy, 1972). In this respect our failure to detect viral sensitivity by HI and SN tests after serial egg passages of the Alice strain may be considered as an additional indication of its purity.

The results obtained in our studies on the growth capacity at high temperatures are in accordance with those of Willers & Beare (1970) who reported a higher virus yield at 40° C. for several resistant strains, compared with their sensitive parents. This marker was also exhibited by reisolates from human vaccinees.

The degree of sensitivity to amantadine of influenza A varies considerably from strain to strain; comparative tests have shown that PR/8 strain was the least sensitive among a series of type A influenza strains (Schild & Sutton, 1965).

Our results indicate that A/England/42/72 was more sensitive to amantadine than the PR/8/34 strain and the MRC-2 recombinant. The Alice strain retained this property. Since no controlled selective pressure favouring the emergence of amantadine resistance variants (Cochran, Maassab, Tsunoda & Berlin, 1965; Lavrov, Podchernyayeva, Blinova & Sokolov, 1972) has been applied during the recombination, the low sensitivity of the MRC-2 strain may have been acquired through genetic exchange (Kutinova, Tuckova, Vonka & Starek, 1971).

Hamsters have been shown to be susceptible to influenza virus (Mills, van Kirk, Hill & Chanock, 1969). Transmission of the Alice strain occurred to one uninoculated contact animal whereas the other non-inoculated animal and one animal inoculated with 10^3 EID 50 dose were not infected although some of their cagemates were excreting virus for as long as 7 days.

The low spreading potential of resistant strains in man and animals was previously reported by Lamy *et al* (1973) and Huygelen *et al.* (1973). Our results in this study seem to indicate that this spreading capacity is higher in hamsters than in humans. This difference must be attributed to the high percentage of hamsters that excrete virus, to the long-excretion period and to the relatively high infectivity

of the strain for hamsters. In the human species, virus excretion is much less frequent and of shorter duration. Also, the virus excreted by human subjects has a very low titre and, in contrast with hamsters, very high titres are required to infect human subjects. This non-communicability in the human species was confirmed by transmission studies based upon virological and serological monitoring of susceptible subjects living in close contact with vaccinees (Rubin *et al.* 1974; Noble *et al.* 1974).

Based upon the study of three of the five criteria established in ferrets by Potter *et al.* (1972) our results demonstrated that the Alice strain was attenuated and immunogenic in ferrets. The Alice strain did not cause any temperature increase in contrast to wild type viruses. The seroconversion rate was similar for the Alice strain and wild type viruses.

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