Antiviral Research, 3 (1983) 241–252
Elsevier

In vivo and in vitro hamster models in the assessment of virulence of recombinant influenza viruses

A.W. Heath*, Caroline Addison, M. Ali, D. Teale and C.W. Potter

Department of Virology, University of Sheffield Medical School, Beech Hill Road, Sheffield S10 2 RX, U.K.

(Received 11 April 1983; accepted 12 July 1983)

The virulence of five wild-type influenza A viruses and 14 recombinant viruses, prepared from the cold adapted A/Ann Arbor/6/60 virus and various wild-type viruses, was studied by two methods. Firstly, the viruses were inoculated into hamsters, and the titres present in the lungs and turbinates at 1, 3 and 4 days post-infection were measured. Secondly, the effect of five wild-type and ten recombinant viruses on the ciliated epithelium of in vitro hamster tracheal organ cultures was examined. The results obtained were assessed with reference to the known virulence of the viruses for human volunteers. The results showed that virus strains virulent for man grew to higher titres in hamster lungs and turbinates than attenuated strains; and that virulent strains destroyed the ciliary activity of hamster tracheal organ cultures more quickly and to a greater extent than attenuated strains. Comparison of the results with the known virulence of viruses tested for man suggests that the reduced ability of virus to grow in hamster lung tissue and the relatively little effect on ciliary activity may be used as markers of virus attenuation; however, the growth of virus in hamster turbinates overlaps for virulent and attenuated strains and therefore was not considered a useful marker of virulence.

virulence assessment; influenza virus; hamster

Introduction

Influenza virus strains of potential value as live virus vaccines can be produced rapidly by selection from recombinant pools produced by double infection of cells with wild-type virulent strains and known attenuated strains [18]. The future use of such recombinant strains as vaccines is considered by some authorities to have many advantages over the use of inactivated vaccines: thus, more doses can be produced per egg; the vaccine is given intranasally which may be more acceptable; and live vaccines have been shown to induce a more solid immunity [4,13]. In contrast, live virus vaccines require protracted testing to ensure safety for general use [28], these tests

*To whom correspondence should be addressed.

0166-3542/83/$03.00

include volunteer studies of attenuation, genetic stability, non-transmissibility and immunogenicity, and the time required for completion may be too long for practical purposes, since epidemic influenza virus strains change antigenically at frequent intervals [36].

The development of attenuated influenza vaccines would be considerably facilitated by the identification and acceptance of laboratory methods to measure human virulence, and a number of such models have been investigated. Infection of ferrets [7,8,12], hamsters [3,22] and infant rats [17–21,37] and growth in organ cultures of human and ferret trachea [6,10,23–25] have been suggested, but none has been accepted to date as a satisfactory alternative to volunteer studies, since either correlation with volunteer studies has not been complete [15], or too few strains have been studied to allow conclusions. In the present study, we report the growth of wild-type and recombinant influenza viruses in the lungs and turbinates of hamsters, together with the effect of these viruses on organ cultures of hamster trachea. The results were compared and related to virulence for man as established in volunteer studies, to determine the value of the models in the measurement of human virulence.

Materials and methods

Viruses

Wild-type influenza viruses A/Finland/4/74 (H3N2), A/Victoria/3/75 (H3N2) and A/Alaska/6/77 (H3N2) were kindly supplied by Dr. J.J. Skehel, National Institute for Medical Research, Mill Hill, London; and strains A/Texas/1/77 (H3N2) and A/Scotland/840/74 (H3N2) were available in this laboratory. Cold-adapted recombinant viruses AA-CR31 clones 2, 3, 10 and 12 and AA-CR29 clones 2, 8 and 17 which are recombinants of cold-adapted A/Ann Arbor/6/60-7PI (H2N2) and A/Alaska/6/77, AA-CR33 clones 2 and 3 which are recombinants between A/Ann Arbor/6/60-7PI and A/USSR/90/77 (H1N1), and AA-CR44 clones 6-1, 11-1, 16-1 and 20-2 which are all recombinants of A/Ann Arbor/6/60-7PI with A/Beijing/2/79 (H3N2) were prepared by Dr. H.F. Maassab, University of Michigan, School of Public Health, Department of Epidemiology. Strains AA-CR19 and AA-CR22 clones 1 and 17 which are all recombinants of A/Ann Arbor/6/60-7PI and A/Victoria/3/75 (H3N2) were supplied by Dr. A. Kendal, Center for Infectious Disease, Centers for Disease Control, Atlanta, Georgia.

Virus pools were prepared by the allantoic inoculation of 10-day embryonated eggs with 0.2 ml 10^{-3} dilution of seed virus. After incubation for 3 days at 34°C, the allantoic fluids were harvested and stored at -80°C and the titre of each pool determined by titration by the allantois-on-shell method [11]: the 50% egg-bit infectious doses ($EBID_{50}$) were calculated using the method of Reed and Muench [33].

Animal inoculation

Eight- to 12-wk-old Syrian hamsters were obtained from a randomly-bred closed

colony at the University of Sheffield. Animals were weaned at age 3 wk and used at age 6–8 wk. Hamsters were lightly anaesthetised with ether and inoculated intranasally with $10^{4.0}$ $EBID_{50}$ of virus in a volume of 0.2 ml of phosphate buffered saline, pH 7.2 (PBS), containing 1% (v/v) BSA and antibiotics: nine hamsters were inoculated with each strain of virus. At 24, 72 and 96 h post-inoculation groups of three animals were killed by cervical dislocation: the lungs and turbinates were removed aseptically, washed with PBS, and separately ground with carborundum powder in PBS containing 2% (v/v) BSA and antibiotics to give a 40% (w/v) suspension. The extracts were then centrifuged at 2 000 $\times g$ for 15 min, and the supernatants stored at -80°C prior to virus titration.

Virus titrations

Extracts of lungs and turbinates from virus-infected hamsters were titrated for virus by the allantois-on-shell method [11]. Each virus dilution was inoculated onto allantois-on-shell from four different eggs since eggs differ in sensitivity to influenza virus infection. After incubation for 3 days at 33°C with constant shaking, shell fragments were removed, and the culture fluids were tested for virus by haemagglutination. The virus titres, expressed in 50% egg bit infectious doses ($EBID_{50}$)/ml were calculated by the method of Reed and Muench [33].

Preparation of tracheal rings

Hamsters were anaesthetised with carbon dioxide then dissected, cutting longitudinally through the sternum and anteriorly to the mandible. The skin was pulled clear and the animal exsanguinated by cardiac puncture. The lungs and trachea were aseptically removed into pre-warmed (37°C) medium 199. The trachea was freed from the lungs in medium 199; washed and rings cut transversely on a sterile surface using a scalpel or razor blade; 8–12 rings were obtained from each trachea by this method. The rings were put separately into sterile capped test-tubes containing 1.0 ml of pre-warmed medium 199, containing 5% (v/v) foetal calf serum and antibiotics and allowed to adhere to the side of the tube 15–20 mm from the bottom for 2 h. The tubes were then rolled at 37°C for 24 h; cell cultures were examined at this time and rings which exhibited active and clearly visible ciliary activity were used for virus inoculation.

Virus inoculation of tracheal rings

Rings showing active ciliary activity after 24 h incubation at 37°C were selected. Growth medium was decanted and they were inoculated with 10^5 $EBID_{50}$ of virus in a 1.0 ml volume and incubated for 2 h at 37°C for virus adsorption to occur. After adsorption the medium was decanted, the cells washed three times with growth medium, and 1.0 ml of fresh growth medium added. The cultures were incubated further for 10–12 days. The ciliary activity of the cultures was assessed daily, prior to changing the medium.

To assess ciliary activity, the tubes were held horizontally under the lens of a microscope with the tracheal ring uppermost; activity was graded under the 40× objective. The ciliary activity at the time of virus inoculation was taken as 100%, and the areas where cilia were beating strongly were marked on a diagram of each ring; later ciliary activity was graded with reference to these diagrams as 100, 75, 50, 25 or 0% of the original.

Results

Virus growth in animal tissues

Wild-type viruses

The replication of wild-type strains of influenza A virus in the turbinates and lungs of hamsters was determined at 1, 3 and 4 days post-inoculation with $10^{4.0}$ $EBID_{50}$ of virus: virus titres were determined in tissue extracts from three animals at each time and for each virus. The results are shown in Table 1. For three of the virus strains, the titres in the turbinate tissue increased with time: however, for influenza virus A/Texas/77, peak titres were found at 24 h post-inoculation, and subsequently declined. The maximum titres observed were $10^{1.7}$–$10^{3.45}$ $EBID_{50}$/ml (Table 1). Virus titres in lung extracts were higher than in turbinates. For three of the virus strains tested, lung titres increased to a maximum at 4 days post-inoculation; but for influenza A/Texas/77 and A/Finland/74 peak titres were observed at 24 h after virus infection. The maximum titres recorded varied from $10^{4.35}$–$10^{6.36}$ $EBID_{50}$/ml (Table 1).

Recombinant viruses

The replication of 14 cold-adapted, recombinant viruses in the lungs and turbinates of hamsters was measured in the same way as described for wild-type viruses. The results are shown in Table 2. Virus titres in the turbinates of infected animals were relatively low; virus was detected in tissue extracts from animals inoculated with 10 of the viruses tested, but not for strain CR29 clones 8 and 17, CR44 clone 6-1 and CR19. For one strain (CR31 clone 3) peak titre occurred at 24 h post-inoculation, whilst for the rest peak titres were found on days 3 and 4 after virus inoculation. Peak titres varied from $<10^{1.0}$–$10^{1.7}$ $EBID_{50}$/ml (Table 2). Virus was recovered from lung extracts for 13 of the 14 virus strains inoculated; for strain CR29 clone 8 no virus was detected in lung or turbinate extracts; whilst for viruses CR19, CR29 clone 17 and CR44 clone 6-1 virus was recovered from lung extracts, but not from the turbinates of infected animals. Peak titres on variable days post-inoculation for the virus tested varied from $<10^{1.0}$-$10^{2.58}$ $EBID_{50}$/ml, and tended to be higher than for turbinate extracts.

Virus effect on hamster trachea ciliary activity

Wild-type viruses

Virulent virus strains A/Texas/1/77, A/Victoria/3/75, A/Scotland/840/74,

TABLE 1

Replication of wild-type virus strains of influenza A in hamster lungs and turbinates

Virus strain	Mean $\log_{10}$ $EBID_{50}$ (± SD)					
	Turbinates			Lungs		
	1 day	3 days	4 days	1 day	3 days	4 days
A/Victoria/3/75	<1.0	1.3 ± 0.2	2.3 ± 0.2	3.0 ± 0.23	3.18 ± 0.84	4.35 ± 0.4
A/Alaska/6/77	1.4 ± 0.3	1.9 ± 0.2	2.8 ± 0.1	<1.0	3.5 ± 0.32	4.66 ± 0.05
A/Scotland/840/74	1.76 ± 0.02	2.74 ± 0.05	3.45 ± 0.24	4.1 ± 0.79	3.9 ± 0.56	4.79 ± 0.56
A/Texas/1/77	2.62 ± 0.6	1.52 ± 0.6	1.17 ± 0.4	6.36 ± 1.66	4.45 ± 0.49	2.8 ± 0.69
A/Finland/4/74	1.09 ± 0.24	1.17 ± 0.46	1.7 ± 0.3	4.86 ± 0.47	3.11 ± 0.19	3.07 ± 0.25

TABLE 2

Replication of recombinant virus strains of influenza A in hamster lungs and turbinates

Virus strains	Mean $\log_{10}$ $EBID_{50}$/ml ± SD					
	Turbinates			Lungs		
	1 day	3 days	4 days	1 day	3 days	4 days
CR 19	<1.0	<1.0	<1.0	2.46 ± 0.6	2.58 ± 0.3	2.3 ± 0.4
CR 22 clone 1	1.2 ± 0.3	<1.0	1.7 ± 0.1	1.2 ± 0.4	<1.0	1.69 ± 0.1
CR 29 clone 8	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0
CR 29 clone 17	<1.0	<1.0	<1.0	<1.0	2.42 ± 0.57	<1.0
CR 31 clone 2	<1.0	<1.0	1.9 ± 0.1	1.0 ± 0.45	2.1 ± 0.1	<1.0
CR 31 clone 3	1.3 ± 0.3	<1.0	1.1 ± 0.3	<1.0	1.76 ± 0.3	<1.0
CR 31 clone 10	<1.0	1.2 ± 0.2	1.2 ± 0.2	1.3 ± 0.2	1.6 ± 0.2	1.3 ± 0.2
CR 31 clone 12	<1.0	1.6 ± 0.3	1.2 ± 0.2	1.1 ± 0.1	1.8 ± 0.2	2.1 ± 0.4
CR 33 clone 2	<1.0	1.3 ± 0.05	<1.0	1.74 ± 0.25	1.9 ±0.33	1.53 ± 0.12
CR 33 clone 3	<1.0	1.2 ± 0.4	1.1 ± 0.1	1.6 ± 0.2	1.8 ± 0.2	1.9 ± 0.3
CR 44 clone 6-1	<1.0	<1.0	<1.0	1.1 ± 0.1	1.6 ± 0.3	1.8 ± 0.2
CR 44 clone 11-1	<1.0	1.1 ± 0.2	1.1 ± 0.2	1.8 ± 0.3	2.3 ± 0.2	1.9 ± 0.2
CR 44 clone 16-1	<1.0	1.1 ± 0.3	1.0 ± 0.2	1.4 ± 0.75	2.11 ± 0.24	<1.0
CR 44 clone 20-2	<1.0	1.3 ± 0.2	<1.0	1.2 ± 0.3	2.1 ± 0.2	2.1 ± 0.2

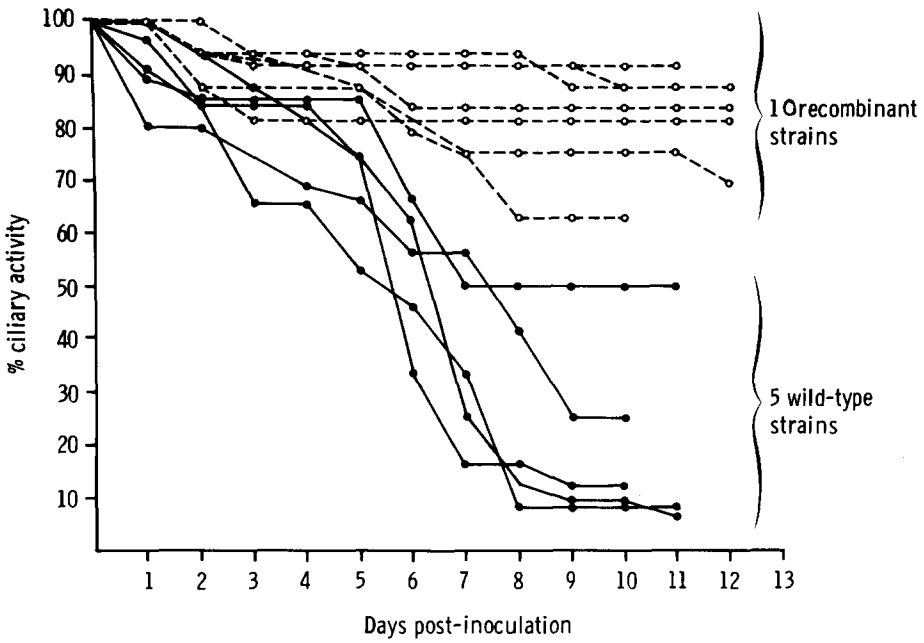

Fig. 1. The action on ciliary activity of 5 wild-type and 10 recombinant influenza virus strains. CR29 clone 2 is included and was not tested in vivo.

A/Alaska/6/77 and A/Finland/4/74 were tested for their effects on the ciliary activity of hamster tracheal organ culture. The results are shown in Fig. 1. All five strains of virus tested had a strongly inhibiting effect on ciliary activity, but the extent of this effect varied for the different strains. Thus, at 10 days following inoculation with A/Finland/74 ciliary activity was reduced by 50%, whilst for A/Texas/77 almost all activity had been lost by this time. For the five strains of virus tested $\geqslant$50% reduction of ciliary activity was recorded at 6–8 days, post-inoculation.

Recombinant viruses

The effect of recombinant viruses on the ciliary action of tracheal organ cultures was measured. The results are shown in Fig. 1. Some variation was observed, but generally the findings were remarkably consistent. Thus, at 6–8 days post-inoculation, the reduction in ciliary action was 62.5–100%, and none of the viruses tested induced 50% reduction in ciliary activity during the observation period (Fig. 1).

Gene origin of recombinant viruses

The gene derivations of the recombinant viruses derived from A/Ann Arbor/6/60-7PI and a wild-type virus have been established (see review [18]). For 6 of the 14 recombinant viruses tested the HA and NA were derived from the wild-type parent and all other genes from the cold-adapted parent. One strain, CR44 clone 20-2, derived only the HA from the wild-type parent. The gene derivation of CR33 clone 3 is

unknown to us and the remaining recombinants possess single, additional, wild-type genes.

These results suggest that those recombinants possessing only the HA and NA from the wild-type parent, and the recombinants with additional wild-type genes grow to similar titres in hamster lungs and turbinates, and that the effect of these two groups of viruses on ciliary activity is also similar. The growth of wild-type viruses in hamster tissue, and their effects on ciliary activity was markedly different from those of the recombinant strains. Thus, peak titre in the lung was $10^{4.35}$–$10^{6.36}$ $EBID_{50}$/ml for wild-type viruses compared to $<10^{1.0}$–$10^{2.58}$ $EBID_{50}$/ml for recombinant strains: and peak titre of virus in the turbinates was $<10^{1.0}$–$10^{1.9}$ $EBID_{50}$/ml for recombinant strains and $10^{1.7}$–$10^{3.45}$ $EBID_{50}$/ml for wild-type viruses. In addition, at nine days post-inoculation of hamster tracheal cultures, the ciliary activity had been reduced by $\geqslant$50% of the original activity (Fig.1).

Correlation of in vitro tests with virus virulence

Influenza viruses A/Victoria/75, A/Scotland/74, A/Alaska/76, A/Texas/77 and A/Finland/74 are past epidemic strains, and are virulent for man: 10 of the 14 recombinant viruses used in the present study have been shown to be attenuated in volunteer studies [9,16,26–29]. The CR44 strains have not been shown to be attenuated in humans. A comparison of the experimental results obtained in the present study with virus virulence for man is shown in Fig. 2. The maximum titres of virus found in

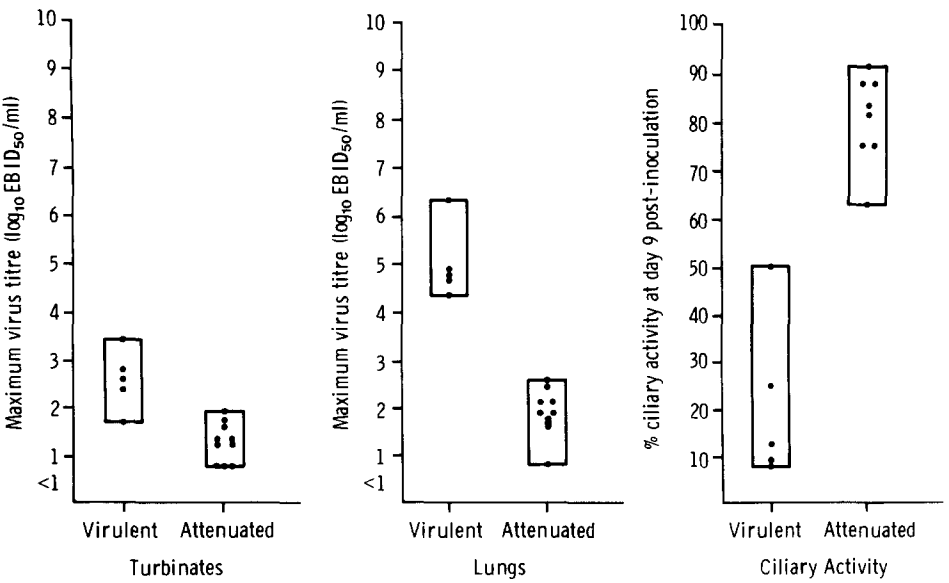

Fig. 2. Correlation of maximum virus titres in hamster lungs and turbinates, and ciliary activity of hamster tracheal rings at 9 days post-inoculation, with virus virulence for man.

turbinate extracts tended to be higher for virulent viruses than for attenuated strains; however, some overlap of results was observed, and for this reason the measurement cannot be considered a reliable measure of human virulence. In contrast, the maximum titre of virus present in lung extracts was $>10^4$ for virulent strains, $<10^3$ for attenuated strains; this suggests that virus growth in this tissue can be used to discriminate virulent from attenuated virus strains. Similarly, the ability of virus strains to inhibit ciliary activity discriminated the two virus groups. The results suggest that the two latter tests could be of value in the laboratory recognition of virulent and attenuated recombinant influenza viruses.

Discussion

Many authorities have suggested that a live attenuated influenza virus vaccine would have advantages over the presently available inactivated vaccines; however, the development of a live vaccine for general use requires the selection of a recombinant of an attenuated parent and the current wild-type strain which is attenuated for man, and the completion of a programme of volunteer studies to show the strain to be genetically stable, non-reactive, non-transmissible and immunogenic; it has been estimated that this would take some two years, by which time the vaccine would not be relevant to the current epidemic virus strains. The period required for developmental studies could be considerably shortened if animal models to assess genetic stability, non-transmissibility and attenuation were developed and accepted, and would make the development of a live influenza vaccine a practical possibility.

A number of model systems have been investigated to extablish a method of measuring in vivo virulence for man. These include infection of ferrets [7,8,12], mice, and infant rats [17,19–21,37]. In addition, virus growth in hamsters and the effects of virus on hamster organ cultures have been studied [1,3,22]. None of these methods enjoys general acceptance as a reliable marker of virus virulence, whilst some methods have not been studied sufficiently to allow assessment of reliability and value. In the present study, 15 influenza A virus strains of known virulence for man were tested for growth in hamster lungs and turbinates, and for reduction of ciliary activity in hamster trachea organ cultures. The results indicate that wild-type virulent strains grew to higher titres in hamsters and reduced ciliary activity to a greater extent than attenuated virus strains: the results were clearly distinct for the two groups of virus, and there was no overlap in the results. Although both the CR and the wild-type viruses used are egg-passages beyond viruses shown to be attenuated and virulent for man respectively, it is highly unlikely that a uniform spontaneous mutational event leading to attenuation of all CR viruses could have occurred.

Using a different series of wild-type and recombinant viruses based on A/PR/8/34 or A/Okuda/57 attenuated strains, Abou-Donia et al. found no correlation between growth in hamsters and virulence for man [1]. In addition, virus virulence for infant rats clearly distinguished wild-type viruses from attenuated strains derived by recombination with A/AA/6/60, but not recombinant strains derived from A/PR/8/34 [2,17].

The results suggest that virus growth in hamster lungs and virus reduction of ciliary activity in vitro are reliable tests for human virulence with wild-type viruses and recombinants of cold-adapted A/Ann Arbor/6/60 virus. The results agree with those of studies in infant rats which also clearly discriminate between the two groups of virus [2]. It is probable however, that the technique cannot be extended to other recombination systems, and this has been demonstrated [1].

In addition, similar results were obtained with virus strains which contained only the genes for the two surface antigens, and those which contained another gene from the wild-type parent. The effect of other wild-type genes on growth in hamsters could not be assessed from the limited number of varieties available for the present study.

Since the development of suitable in vivo models for establishing the properties of putative influenza vaccine strains may be necessary for practical purposes, and many results indicate that vaccines based on recombinants of wild-type and cold-adapted A/Ann Arbor/60 may be the best choice, we suggest that the hamster model outlined above merits further testing to determine reliability and value as a model of attenuation. The test could be used in conjunction with other in vivo methods of measuring virus virulence, and facilitate in the development of live attenuated influenza virus vaccines.

Acknowledgement

The authors would like to thank the Medical Research Council for a grant enabling these studies to be carried out.

References

1 Abou-Donia, H., Jennings, R. and Potter, C.W. (1980) Growth of influenza A viruses in hamsters. Arch. Virol. 65, 99–107.

2 Ali, M., Maassab, H.F., Jennings, R. and Potter, C.W. (1982) Infant rat model of attenuation for recombinant influenza viruses prepared from cold-adapted attenuated A/Ann Arbor/6/60. Infect. Immun. 38, 610–619.

3 Ali, M.J., Teh, C.Z., Jennings, R. and Potter, C.W. (1982) Transmissibility of influenza viruses in hamsters. Arch. Virol. 72, 187–197.

4 Beare, A.S., Hobson, D., Reed, S.E. and Tyrell, D.A.J. (1968) A comparison of live and killed influenza virus vaccines. Lancet ii, 418–420.

5 Blaskovic, P., Rhodes, A.J. and Labzoffsky, N.A. (1972) Infection of chick embryo tracheal organ cultures with influenza A2 (Hong Kong) virus. Arch. Gesamte Virusforsch. 37, 104–113.

6 Boudrealt, A. (1979) Live influenza vaccine; screening of attenuated virus strains by 50% ciliary activity inhibition test in organ cultures of ferret trachea. Can. J. Microbiol. 25, 279–284.

7 Campbell, D., Sweet, C. and Smith, H. (1979) Comparisons of virulence of influenza virus recombinants in ferrets in relation to their behaviour in man and genetic constitution. J. Gen. Virol. 44, 37–44.

8 Campbell, D., Sweet, C., Hay, A.J., Douglas, A., Skehel, J.J., Mason, T.J. and Smith, H.

(1982) Genetic composition and virulence of influenza virus: Differences in facets of virulence in ferrets between two pairs of recombinants with RNA segments of the same parental origin. J. Gen. Virol. 58, 387–398.

9 Cate, J.R. and Couch, R.B. (1982) Live influenza A/Victoria/3/75 (H3N2) virus vaccines, immunogenicity and protection against wild-type virus challenge. Infect. Immun. 38, 141–146.

10 Diaz-Rodriguez, P. and Boudrealt, A. (1982) Inhibition of ciliary activity in organ cultures of ferret trachea in reference to genetic and biological characters of influenza virus strains. Can. J. Microbiol. 28, 809–814.

11 Fazekas de St. Groth, S., Witchell, S.J. and Lafferty, K.J. (1958) An improved assay for infectivity of influenza virus. J. Hyg. 56, 151.

12 Fenton, R.J., Jennings, R. and Potter, C.W. (1977) Differential response of ferrets to infection with virulent and avirulent influenza viruses: A possible marker of virus attenuation. Arch. Virol. 55, 55–66.

13 Freestone, D.S., Hamilton-Smith, S., Buckland, R., Chinn, S. and Tyrrell, D.A.J. (1972) Antibody responses and resistance to challenge in volunteers vaccinated with live attenuated, detergent split and oil adjuvant A2/Hong Kong/68 (H3N2) influenza vaccines. J. Hyg. 70, 531–543.

14 Freestone, D.S., Bowker, C.H., Letley, E., Ferris, R.D., White, W.G. and Barnes, G.M. (1976) A clinical trial of WRL105 strain live attenuated influenza vaccine comparing four methods of intranasal vaccination. J. Hyg. (Cambridge) 76, 459–466.

15 Hara, K., Beare, A.S. and Tyrrell, D.A.J. (1973) Growth and pathogenicity of influenza viruses in organ cultures of ciliated epithelium. Arch. Gesamte Virusforsch. 44, 227–236.

16 Hrabar, A., Vodopija, I., Andre, F.E., Mitchell, J.R., Maassab, H.F., Hennessy, A.V. and Davenport, F.M. (1977) A placebo controlled dose-response study of reactogenicity and immunogenicity of a cold-adapted recombinant A/Victoria/3/75 (H3N2) live influenza virus candidate vaccine in healthy volunteers. Dev. Biol. Stand. 39, 53–60.

17 Jennings, R., Potter, C.W., Teh, C.Z. and Mahmud, M.I.A. (1980) The replication of type A influenza viruses in the infant rat. A marker for virus attenuation. J. Gen. Virol. 49, 343–354.

18 Kendal, A.P., Maassab, H.F., Alexandrova Galina, I. and Ghendon, Y.Z. (1981) Development of cold-adapted recombinant live, attenuated influenza A vaccines in the U.S.A. and U.S.S.R. Antiviral Res. 1, 339–365.

19 Mahmud, M.I.A., Jennings, R. and Potter, C.W. (1979) The infant rat as a model for assessment of the attenuation of human influenza viruses. J. Med. Micro. 12, 43–54.

20 Mahmud, M.I.A., Maassab, H.F., Jennings, R. and Potter, C.W. (1979) Influenza virus infection of newborn rats: virulence of recombinant strains prepared from a cold-adapted, attenuated parent. Arch. Virol. 61, 207–216.

21 Michaels, R.H., Mahmud, M.I.A., Coup, A., Jennings, R. and Potter, C.W. (1978) Influenza virus infection in newborn rats: a possible marker of attenuation for man. J. Med. Virol. 2, 253–264.

22 Mills, J. and Chanock, R.M. (1971) Temperature sensitive mutants of influenza virus. I. Behaviour in tissue culture and in experimental animals. J. Inf. Dis. 123, 145–157.

23 Mostow, S.R. and Tyrrell, D.A.J. (1973) The behaviour in vitro of attenuated recombinant influenza viruses. Arch. Gesamte Virusforsch. 43, 385–392.

24 Mostow, S.R., Flataver, S., Paler, M. and Murphy, B.R. (1977) Temperature sensitive mutants of influenza virus. XIII. Evaluation of influenza A/Hong Kong/68 and A/Udorn/72 ts and wild type viruses in tracheal organ culture at permissive and restrictive temperatures. J. Inf. Dis. 136, 1–6.

25 Mostow, S.R., Hopkins, J. and Wright, P.F. (1979) Behaviour of vaccine revertants of temperature-sensitive mutants of influenza virus in ferret tracheal organ culture. Infect. Immun. 26, 193–196.

26 Moritz, A.J., Kunz, C., Hofman, H., Liehl, E., Reeve, P. and Maassab, H.F. (1980) Studies with a cold recombinant A/Victoria/3/75 (H3N2) virus. II. Evaluation in adult volunteers. J. Inf. Dis. 142, 857–860.

27 Murphy, B.R., Wood, F.T., Massicot, J.G. and Chanock, R.M. (1978) Temperature sensitive mutants of influenza virus XVI. Transfer of two ts lesions present in the Udorn/72-ts-IA_2 donor virus to the Victoria/3/75 wild-type virus. Virology 88, 244–251.

28 Murphy, B.R., Holly, Jr., H.P., Berquist, E.J., Levine Myron, M., Spring, S.B., Maassab, H.F., Kendall, A.P. and Chanock, R.M. (1979) Cold-adapted variants of influenza A virus: evaluation in adult seronegative volunteers of A/Scotland/840/74 and A/Victoria/3/75. Cold-adapted recombinants derived from the cold-adapted A/Ann Arbor/6/60 strain. Infect. Immun. 23, 253–259.

29 Murphy, B.R., Chanock, R.M., Clements, M.L., Anthony, W.C., Sear, A.J., Cisneros, L.A., Rennels, M.B., Miller, E.H., Black, R.E. Levine, M., Betts, R.F., Gordon, Jr., D.R., Maassab, H.F., Cox, N.J. and Kendal, A.P. (1981) Evaluation of A/Alaska/6/77 (H3N2) cold-adapted recombinant viruses derived from A/Ann Arbor/6/60 cold adapted donor virus in adult sero negative volunteers. Infect. Immun. 32, 693–697.

30 Murphy, B.R., Hinshaw, V.S., Sly, D.L., London, W.T., Hosier, N.T., Wood, F.T., Webster, R.G. and Chanock, R.M. (1982) Virulence of influenza A viruses for squirrel monkeys. Infect. Immun. 37, 1119–1126.

31 Potter, C.W., Oxford, J.S., Shore, S.L., McLaren, C. and Stuart-Harris, C.H. (1972) Immunity to influenza in ferrets. I. Response to live and killed virus. Brit. J. Exp. Pathol. 53, 153–167.

32 Potter, C.W. and Jennings, R. (1976) The hamster as a model system for the study of influenza vaccines. Postgrad. Med. J. 52, 345–351.

33 Reed, L. and Muench, H.A. (1938) A simple method of establishing 50% end points. Am. J. Hyg. 27, 493–497.

34 Reeve, P., Geredas, B. and Walz, H. (1978) Growth of some attenuated influenza viruses in hamster tracheal organ cultures. Med. Microbiol. Immunol. 166, 141–150.

35 Schiff, L.J. (1974) Studies on the mechanisms of influenza virus infection in hamster tracheal organ culture. Arch. Gesamte Virusforsch. 44, 195–204.

36 Stuart-Harris, C.H. and Schild, G.C. (1976) Influenza: The viruses and the disease. Edward Arnold, London, ch. 3.

37 Teh, C.Z., Jennings, R. and Potter, C.W. (1980) Influenza virus infection of newborn rats: virulence of recombinant strains prepared from influenza virus strain A/Okuda/57. J. Med. Microbiol. 13, 297–306.