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Growth of Some Attenuated Influenza Viruses in Hamster Tracheal Organ Cultures

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Abstract. Hamster tracheal organ cultures were infected with different influenza viruses and the metabolic activity measured using a tetrazolium reduction assay. In addition, relative ciliary activity was observed, virus multiplication measured, and histological studies were made. The hamster organ cultures proved relatively simple to set up and were sensitive to infection with influenza viruses; and the tetrazolium reduction was a reliable objective measure of relative tissue damage correlating well with histological and virological findings as well as with visual assessment of ciliary activities.

Introduction

In recent years, there has been considerable activity in the development of live attenuated influenza vaccines. Current developments utilize essentially the same basic approach: recombination of an attenuated basal strain (the donor strain) with a wild-type virus possessing the required surface antigens of a current epidemic serotype. Subsequent procedures allow the selection of hybrid viruses possessing the attenuation of the donor strain with the antigenic potential desired.

Currently it is found desirable that donor strains should have well-defined genetic markers linked to their attenuation for man. Several donor strains obtained by widely different techniques are now under investigation as suitable for the development of live influenza vaccines. The commercially available Gripovax vaccine is based on a strain, RIT 4050, derived by recombination with AoPR8 and selected on the basis of resistance to nonspecific inhibitors (Hugelyn, 1977). In the United States two research groups, Maassab et al. (Ann Arbor, Michigan) and Murphy et al. (National Institutes of Health, Bethesda) have derived attenuated strains in which definable genetic lesions have been introduced either by the process of 'cold adaptation', (Maassab, 1969; Maassab et al., 1969; Maassab, 1970; Maassab et al., 1972) or using chemical mutagens to induce temperature sensitive (*ts*) lesions in the viral genome (Mills and Chanock, 1971; Murphy et al., 1976). Both, the cold-adapted influenza viruses and the *ts* viruses from NIH have been shown to be genetically stable and to bear defined genetic lesions capable of transfer

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to influenza viruses of different antigenic subtype (Richman et al., 1975; Spring et al., 1975a; Spring et al., 1975b). In addition, clinical studies have shown these viruses and hybrids derived therefrom to be attenuated for man, inducing only low levels of disease in volunteers (Mills et al., 1969; Beare et al., 1971; Edwards et al., 1972; Murphy et al., 1972; Murphy et al., 1973; Murphy et al., 1974).

Thus, there are at present several promising developments in live influenza vaccines all of which have been shown by human studies to possess some degree of attenuation. Although the final demonstration of attenuation for man must depend on the outcome of controlled clinical trials with human volunteers, in vitro or in vivo tests able to evaluate the virulence of candidate vaccine strains are clearly highly desirable. In previous studies tracheal organ cultures from human embryos or from ferrets have been used to measure the relative virulence of some influenza strains (Mostow et al., 1974; Mostow and Tyrrell, 1973), using subjective visual assessments of relative ciliary activities as a measure of tissue viability. More recently the hamster has been shown to be highly susceptible to infection with respiratory viruses including influenza viruses (Mills and Chanock, 1971; Jennings et al., 1977a; Jennings et al., 1977b; Reeve, 1977). In the following publication we have compared attenuated influenza viruses bearing similar surface antigens but derived from different attenuated donor strains in hamster tracheal organ cultures.

In addition to visual assessment of ciliary activity, we have investigated the use of a tetrazolium reduction assay and compared these results with histological and virological findings.

Materials and Methods

The following virus strains were used:

1. A2/Eng/42/72 H3N2 (A2-Eng). A wild-type virus (A2-Eng) obtained from: WHO Influenza Center for Medical Research (London).
2. A2/Vic/3/75 H3N2 (A2-Vic). A wild-type virus obtained from WHO Influenza Center (London).
3. A2/Vic/75/ts-1 (E) H3N2 clone 81 (ts-1[E]) obtained from B. Murphy (NIH, Bethesda, USA).
4. A2/Vic/75/CR22 H3N2 NIH clone 1 (CR22) obtained from H. Maassab (Ann Arbor, Mich., USA).
5. A2/Vic/3/75 H3N2 RIT 4050 (RIT 4050). The vaccine strain was purchased as a freeze-dried preparation suitable for human inoculation.
6. A_O/PR/8/34 obtained from M. Coleman (CDC, Atlanta, USA).

Virus pools were prepared in 10-day-old specific-pathogen free (SPF) eggs, the virus-infected allantoic fluids being harvested at times known to produce optimal yields. All pools were shown to be bacteriologically sterile and to be free from mycoplasma.

Virus Titrations: Hemagglutinin titrations were made by the microtiter method using phosphate-buffered (PBS) pH 7.2 as a diluant and 0.5% chicken red blood cells. Virus infectivity titrations were made in 10-day-old fertile hens' eggs inoculated allantoically with 0.2 ml volume serially diluted virus using PBS as diluant. Titers were calculated

using the tables given in Meynell and Meynell (1970) and expressed as 50% eggs infective doses (EID_{50}) per 0.2 ml.

Infectivity titrations in MDCK cells were made in the presence 10 μgm/ml trypsin (Difco 1:250) using Eagle's MEM. Titers were expressed as 50% tissue culture infective doses ($TCID_{50}$) or as plaque-forming units (PFU).

Tracheal Organ Cultures: Specific-pathogen free hamsters, 3- to 4-weeks old, were killed with interperitoneal pentabarbitone, and the trachea excised. After washing several times in PBS, serial sections, approximately 1–2 mm thick, were cut from the trachea using scissors, and incubated in Eagle's MEM.

Assessment of Ciliary Activity and cellular viability: Ciliary activity was assessed visually using transmitted light and an inverted microscope. The activity was assessed arbitrarily with reference to uninfected control cultures. For metabolic activity a tetrazolium reduction assay was used (Gabridge and Polisky, 1976). The optical density at 490 nm (OD 490) of acetone-extracted material was determined spectrophotometrically and expressed in relation to the dry weight of the tracheal rings.

For histological examinations tracheal rings were fixed in Bouin's fluid for 24 h, washed with 70% (v/v) ethyl alcohol using methylbenzoate as intermediate and sectioned after embedding in paraffin. Alcian blue haematoxylin eosin sections were examined and assessed histologically, and the number of ciliated cells per 100 μ length of epithelial surface recorded.

Results

1. Infection of Hamster Tracheal Organ Cultures with Wild-type Viruses

Uninfected organ cultures survived up to 12 days, the length of the observation period, as shown by the presence of ciliary activity. Histologically tracheal rings retained their structure with little degeneration. Metabolic activity as evidenced by the tetrazolium reduction assay showed a slow decline over the observation periods (Table 1).

When tracheal organ cultures were infected with $10^{2.0}$ EID_{50} per culture of the wild-type virus A2-Eng or A2-Vic, ciliary activity diminished within 2 days and metabolic activity was considerably reduced (Table 1).

Histological examination showed profound disturbances. From the first day after infection onwards, alterations of the epithelium became apparent, considerable losses of cilia occurred, flattening of epithelium was seen, and by 3 and 4 days after infection cellular debris was seen on top of the epithelium surface (Table 2).

Virus multiplication of A2-Eng in tracheal rings reached a peak about 3 days after infection when maximum titers of 4.5×10^4 PFU were produced by each infected culture (Table 1).

The effect of differing multiplications was measured by infecting tracheal rings with virus dilutions and measuring metabolic activities 3 days after infection (Fig. 1). A linear relationship between dose of virus inoculated and metabolic activity was seen over the range tested. As little as one EID_{50} (log 10) induced a measurable reduction in metabolic activity. Ciliary activity, assessed visually diminished in parallel to the loss of metabolic activity.

Table 1. Infection of hamster tracheal organ cultures with influenza virus strain A2-Eng-74, $10^{3.0}$ EID_{50} per culture

Days after infection	Tetrazolium reduction assay O.D. 490 nm/mg dry wt		Virus yield PFU/0.2 ml log 10
	Uninfected cultures	Infected cultures	
0	0.76 ± 0.33	0.45 ± 0.12	<1.0
1	0.81 ± 0.17	0.42 ± 0.13	1.50 ± 0.45
2	0.54 ± 0.12	0.30 ± 0.12	3.39 ± 1.06
3	0.63 ± 0.51	0.28 ± 0.16	4.56 ± 0.17
4	0.42 ± 0.06	0.23 ± 0.12	4.44 ± 0.25
5	0.42 ± 0.26	0.16 ± 0.14	3.75 ± 0.11
6	0.38 ± 0.97	0.14 ± 0.16	3.30 ± 0.48
7	0.40	0.05	1.40

Cultures were incubated at 35°C Results are the mean of four replicate experiments (except those for day 7). In each experiment the results of six replicate cultures were pooled; for the tetrazolium assay, cultures were incubated with 0.04 M sodium succinate 0.003 M 2,3,5-triphenyl tetrazolium chloride in Tyrodes' buffer and acetone-extracted chromogen estimated at 490 nm. Virus yields were assayed in MDCK cells using Eagle's MEM containing 10 μgm trypsin/ml and 0.8% (w/v) Noble agar

Table 2. Histological findings from hamster tracheal organ cultures after infection with influenza viruses, $10^{3.0}$ EID_{50} per culture

Strain	Number of preparation	Days after infection: 1 C	E	D	2 C	E	D	3 C	E	D	4 C	E	D	5 C	E	D	6 C	E	D
																	7th day		
Uninfected	1	8	●	–	4	●	–	4	○	–	10	●	–	12	●	–	5	○	–
	2	8	●	–	10	●	–	4	●	–	7	●	–	7	●	–	11	●	–
A2-Eng	1	0	○	–	2	□	–	2	○	+	6	□	+	0	□	–	*	*	*
	2	0	○	–	3	●	–	2	□	+	0	□	+	2	□	–	*	*	*
A_0PR8	1	8	●	–	*	*	*	7	●	–	3	●	–	*	*	*	*	*	*
CR22	1	2	○	–	9	●	–	10	●	–	9	●	–	7	○	+	4	●	+
	2	5	●	–	10	●	–	10	●	–	9	●	–	5	●	–	0	○	+
	3	6	●	–	5	●	–	9	●	–	8	●	–	5	○	–	5	○	–
RIT 4050	1	3	○	–	4	○	–	5	○	–	3	○	–	8	○	–	*	*	*
	2	7	○	–	5	●	–	6	○	–	*	*	*	6	○	–	*	*	*

At different times after infection tracheal slices were fixed, section stained, and examined. The predominant type of epithelial cell seen, the relative number of ciliated cells counted and the presence of epithelial debris were recorded.

C = Number of ciliated cells per 100 μ epithelial surface
E = Type of predominant epithelial cell ● = columnar, ○ = cuberoidal, □ = squamous
D = The presence (+) or absence (–) of cellular debris on top of the epithelial surface
*Not done

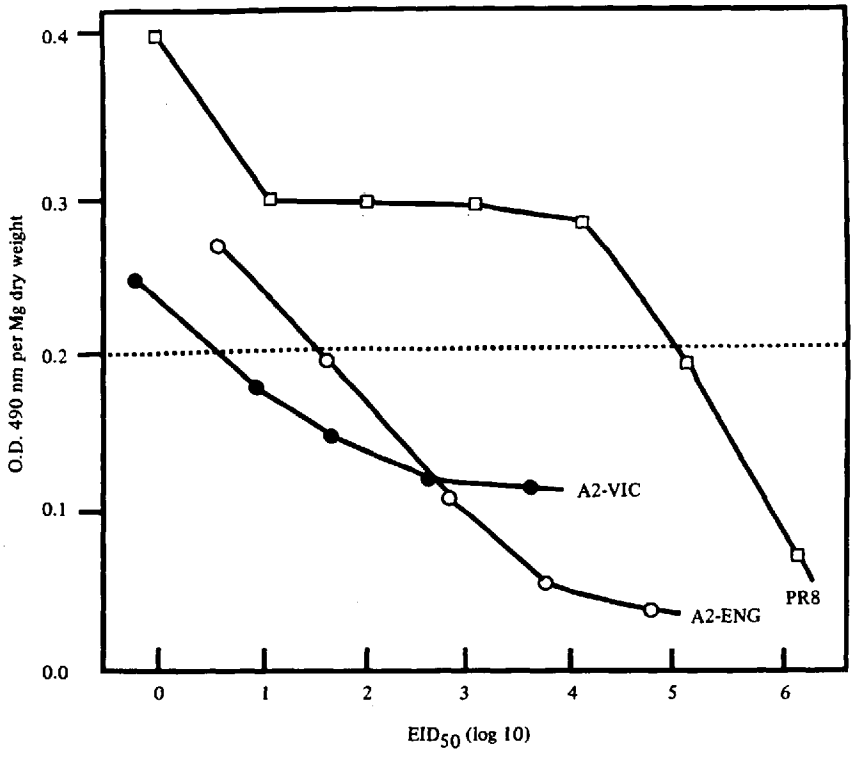

Fig. 1. Infection of hamster tracheal organ cultures with varying doses of influenza viruses. After 3-day incubation at 35°C cultures were incubated for 30 min at 37° C with 0.04 M sodium succinate 0.003 M 2,3,5-triphenyl tetrazolium chloride in Tyrodes' buffer, and the resulting chromogen acetone extracted and assayed at 490 nm. Control uninfected cultures incubated at the same time were assayed for tetrazolium reduction and were found to yield 0.60 ± 0.50 O.D. 490 nm/mg dry weight of tissue. Each point represents the mean value of six replicate cultures

When tracheal rings were infected with different doses of the PR8 strain, a linear reduction in metabolic activity was also seen, but only with relatively high doses of virus. With virus doses up to 10^3 EID_{50} and greater, a marked reduction in metabolic activity was seen paralleling that following infection with strain A2/Eng/74 (Fig. 1). Hamster tracheal rings were thus highly sensitive to infection with wild-type viruses. The pathogenic effect of virus growth could be reliably and objectively estimated using the tetrazolium reduction assay.

2. Infection of Hamster Tracheal Rings with Attenuated Virus Strains

When we investigated the effect of varying virus concentrations on the metabolic activity of hamster tracheal organ cultures 3 days after infection, differences were observed both between wild-type viruses and the different attenuated virus strains. The RIT 4050 strain induced the least metabolic disturbance as judged by tetrazolium reduction assay (Fig. 2).

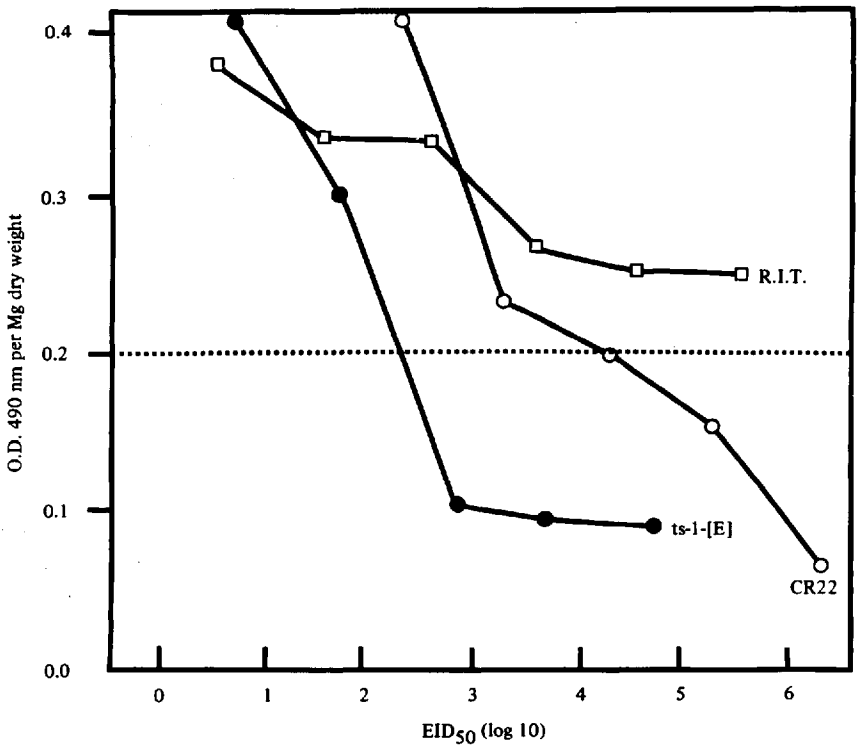

Fig. 2. See legend of Figure 1

With tracheal organ cultures inoculated with virus derived from the ts-1 (E) strain a linear response was seen between dose of virus inoculated and reduction in metabolic activity. Indeed, the reduction in metabolic activity was very close to that seen with the wild-type A2/Eng and A2-Vic strains (Fig. 1). Visual investigation of ciliary activity confirmed these findings (data not shown), which were paralleled by histological findings. The CR22 strain also showed a linear response between dose of virus inoculated and metabolic activity, but appeared to be considerably less virulent than wild-type viruses or *ts*-1 (E) since considerably greater doses were required to induce a comparable reduction of metabolic activity (Fig. 2).

Infection with strain CR22 caused little if any change in the morphology of the epithelium of tracheal organ cultures when compared with noninfected control cultures. The number of ciliated cells within the epithelium was in fact higher than in control cultures. By day 6, however, some cellular debris is found on the epithelial surface. The least alteration in metabolic activity of tracheal rings was induced by strains RIT 4050 and PR8. With these strains relatively high doses were required to induce substantial reductions in metabolic activity. After infection with strain RIT 4050, a minute reduction of the height of epithelium was seen (Table 2), but the number of ciliated cells was still close to that seen in control cultures. Strain A_0/PR/8/34 infected tracheal organ cultures were histologically very similar to those infected with strains CR22 and RIT 4050 (Table 2).

Table 3. Relative virulence of influenza strains for hamster tracheal organ cultures

Strain	Dose reducing metabolic activity 50% (EID_{50} log 10)	Relative virulence for man
A2-Eng	1.7	Unknown, low passage isolate from influenza
A2-Vic	1.0	Virulent, induces influenza in volunteers (Murphy et al., 1977)
A_0-PR8	5.0	Avirulent (Beare et al., 1975)
ts-1 [E]	2.4	Slightly virulent (Murphy et al., 1977)
CR22	4.5	Avirulent (Kunz et al., unpublished observations
RIT 4050	>5.5	Avirulent (Hugelyn, 1977)

Three days after incubation at 35°C the ability to reduce tetrazolium was measured. Results are dose of virus EID_{50} per culture log 10, required to reduce metabolic activity by 50% as compared to uninfected cultures

Control cultures incubated under similar conditions in the absence of virus infection showed some variations in the number of ciliated cells and to a lesser degree some differences in the predominance of columnar or cuboidal epithelium on the inner surface of the trachea. Even 5 to 7 days after culture, numerous healthy ciliated cells were seen in sections.

In summary, gross differences were seen in the metabolic activity of tracheal rings infected with the different influenza viruses. These can be expressed conveniently by comparing the doses required to reduce metabolic activities by 50% (Table 3). From such a comparison the wild-type viruses A2-Eng and A2-Vic are clearly highly virulent for this culture system, small doses of virus ($10^{1.0}$ - $10^{1.7}$ EID_{50}) reducing metabolic activity substantially. The strains PR8 and RIT 4050 were relatively avirulent judged by this criterion, requiring $10^{5.0}$ and at least $10^{5.5}$ EID_{50} respectively to induce comparable metabolic reductions. Strain CR22 behaved as an avirulent strain, 50% reduction was induced by $10^{4.5}$ EID_{50}, but the ts-1 (E) strain was in contrast more virulent, only $10^{2.7}$ EID_{50} reducing metabolic activity by 50% (Table 3).

3. Effect of Temperature

Both *ts*-1 (E) and CR22 possess 'ts' lesions (Spring et al., 1977). Multiplication with *ts*-1 (E) is restricted above 37°C and at 39°C with CR22. We investigated the effect of differing temperatures on the pathogenicity of the 'ts' viruses in hamster organ cultures. As anticipated, virus growth above permissive temperature was markedly impaired and consequently virus-induced pathogenicity reduced. Neither of the temperature-sensitive viruses induced pathological changes as seen in histological sections on reductions in the tetrazolium reduction assay when incubated with tracheal organ cultures above the permissive temperatures.

Discussion

Hamster tracheal organ cultures proved simple to establish and proved to be highly susceptible to infection with influenza virus type A strains. With wild-type virus, especially the A2-Eng strain, virus grew freely in these organ cultures, and concomitently marked changes in metabolic activity and ciliary activity were recorded.

The assay of metabolic activity by the tetrazolium assay proved, as described by Gabridge and Polisky (1976) to be a highly reliable, objective measure of tissue damage paralleling changes seen in ciliary activity observed by light microscopy and subjectively assessed.

Attenuated viruses were investigated to see if organ cultures reflected known differences in the virulence of these strains for man and experimental animals. The RIT 4050 strain is known to be highly attenuated for man (Hugelyn, 1977). In our system it was the most highly attenuated. Relatively small reductions in metabolic activity were observed. Histologically, too, little damage was seen even with relatively high dosages. Strain PR8 behaved similarly and was of very low virulence for the tracheal organ cultures. In man this strain, too, is virtually apathogenic (Beare et al., 1975). In contrast the ts-1 (E) strain has been shown to be less attenuated for man, Murphy et al. (1977) observing clinical reactions in 10% of seronegative human volunteers. In hamsters infected intranasally, this virus multiplies extensively in lungs and nasal tissue and resembles wild-type viruses (Reeve, 1977). Relatively higher doses of ts-1 (E) were, however, required to reduce metabolic activity by 50% (Table 3) as compared to A2-Eng and A2-Vic virus strains.

The cold recombinant virus CR22 was derived by Maassab by recombining the A2/Vic/75 strain and the A/AA/6/60 cold-adapted strain. It is highly attenuated in human volunteers (Kunz et al., unpublished observations). In our organ culture system it was also judged to have low virulence when compared to A2-Eng and A2-Vic. High doses of virus were required to reduce metabolic activity (Table 3) and little tissue damage was seen in histological sections.

In conclusion, we find the hamster organ culture system very simple and the tetrazolium assay of Gabridge and Polisky (1976) gives a simple objective assay of metabolic activity. In addition, hamsters are relatively more easily obtainable and managed than ferrets (Jennings et al., 1977) and can be obtained from SPF sources. When used in comparative dose-response studies difference in virulence can apparently be demonstrated with hamster tracheal organ cultures. The order of virulence for our system (Table 3) followed the order of attenuation in man, but only a small number of strains have been investigated. It remains to be seen if this assay can be of use in predicting the attenuation of future candidate vaccine strains.

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