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Cold-adapted Reassortants of Influenza A Virus: Pathogenicity of A/Ann Arbor/6/60 × A/Alaska/6/77 Reassortant Viruses *In vivo* and *In vitro*

By

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With 1 Figure

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Summary

Cold-adapted reassortants of A/Ann Arbor/6/60 × A/Alaska/6/77 viruses made in MDCK cells have recently been assessed genotypically and for temperature-sensitive and cold-adapted phenotypes. These reassortants were used to infect ferrets and hamsters and to inoculate organ cultures of hamster tracheal rings, in order to assess their degree of virulence. Virulence in the three model systems corresponded quite well, and a correlation between loss of virulence and particular A/AA/6/60 genes present in the reassortants was noted. Two different reassortants containing either RNA 2 or RNA 5 (NA gene) alone from A/AA/6/60 showed little attenuation from the wild-type parent. A reassortant containing both RNA 2 and the NA gene from A/AA/6/60 and all remaining wild-type genes showed some small decrease in virulence compared to the wild-type virus. However a reassortant containing these two A/AA/6/60 genes and RNA 3 as an additional gene from this parent, had a level of attenuation comparable to that of the cold-adapted virus.

Introduction

The cold-adapted influenza virus, A/Ann Arbor/6/60, has been used as an attenuated donor strain in the production of live virus vaccine candidates by reassortment (1, 9, 11, 17). Thus, reassortants have been produced by

mixed infection of primary chick kidney (PCK) cells with cold-adapted (ca) virus and various wild-type (wt) strains. Most of these reassortants have derived only the two surface antigens from the wt parent, while others have a single additional wt protein (8). Viruses with these genotypes retained the phenotypic properties of the attenuated parent, being both ca and temperature sensitive (ts), and were predictably attenuated in man (1, 2, 12, 13). Many attempts have been made to determine the genetic basis of these three properties, but these efforts have largely proved fruitless through lack of reassortants containing a fuller range of genes derived from the wild-type parent (3, 4, 5). However, two different "single gene" reassortants of A/Ann Arbor/6/60 and A/Alaska/6/77 have been isolated from a mixed infection of MDCK cells (14). Also isolated was a "double gene" reassortant containing both the A/AA/6/60 genes of the "single gene" reassortants, and a fourth virus with these two plus a third gene from the ca parent.

Many studies have involved the examination of laboratory models for influenza virus virulence, our studies have shown that the ferret can provide a suitable model system for the assessment of virulence or attenuation of ca reassortants (10); we have also measured the replication of viruses in hamster lungs and turbinates, and performed *in vitro* studies on the effects of reassortant strains on the ciliary activity of hamster tracheal organ cultures: both of these models have proved to be of value, and the results have correlated with experience in man (7). In the present study the four above mentioned reassortants, prepared in MDCK cells, were assessed for virulence as measured by their growth in the lungs and turbinates of ferrets and hamsters, clinical symptoms in ferrets, and the reduction of ciliary activity in hamster tracheal organ cultures. The results obtained have been compared and related to genotypic and phenotypic properties of the virus strains.

Materials and Methods

Viruses

Preparation of the cold-adapted parent virus, A/Ann Arbor/6/60, has been described previously (9). The wild-type strain A/Alaska/6/77 (H 3 N 2) was already available to us, and reassortants of these two strains were prepared by mixed infection of MDCK cells (14). The genetic composition and some phenotypic properties of these viruses have already been reported (14).

Virus pools were grown in embryonated eggs and titrations of virus in allantoic fluid and organ extracts were performed by the allantois-on-shell method of Fazekas de St. Groth *et al.* (6, 7). In addition the 50 per cent egg-infectious dose was determined. Both titres were calculated by the method of Reed and Muench (16).

Genotype and Phenotype of the Viruses

The "single-gene" reassortants, T_4 31-1-1 and T_4 8-7-1-1 possess the RNA 2 and NA genes respectively from the ca parent. The "double-gene" reassortant, T_3 25-1-1, has both of these genes and the "triple gene" reassortant, T_6 6-1-1, possesses in addition the RNA 3

gene from A/AA/6/60. Three of the four viruses are ca and non-ts in MDCK cells, but with the addition of RNA 3 from the ca parent, T_6 6-1-1 becomes ts (15).

Animal Inoculation

Hamsters

Six to eight week-old Syrian hamsters were obtained from the University of Sheffield and were intranasally inoculated with 10^4 $EBID_{50}$ of virus in PBS with 1 per cent BSA and antibiotics. Nine animals were inoculated with each virus and at 24, 72 and 96 hours post-inoculation groups of three animals were killed and their lungs and turbinates removed, washed, and ground with carborundum powder to give a 40 per cent (w/v) suspension in PBS + 2 per cent BSA + antibiotics. This procedure has been described in detail elsewhere (7). The extracts were then clarified by centrifugation and the supernatants stored at −80° C. Virus titre was then assayed by the allantois-on-shell method (6, 7).

Ferrets

Ferrets were obtained from Marshall Research Animals, North Rose, New York 14516, U.S.A. Animals were inoculated at 8 weeks of age with about 10^8 EID_{50} of virus. Animals were killed at 3 and 8 days post-inoculation and 10 per cent suspensions of lung and turbinate tissue were prepared as above for virus titration. Titration was by determination of EID_{50}. In addition, nasopharyngeal swabs were made daily and the rectal temperature was monitored.

Hamster Tracheal Rings

The preparation of hamster tracheal ring organ cultures has already been described (7). Cultures showing active ciliary activity after 24 hours incubation were inoculated with 10^5 $EBID_{50}$ (50 per cent Egg-Bit infectious dose) of virus. Virus adsorption was for 2 hours and then cultures were washed and incubated for 10–12 days on a roller at 37° C. Ciliary activity of the cultures was assessed daily prior to changing the medium. The activity of each culture was graded as 100, 75, 50, 25, or 0 per cent of the original activity (7).

Results

Virus Infection in Ferrets

Table 1 shows the virus titres measured in the lungs and turbinates of ferrets and the clinical response of the animals following infection. The cold-adapted donor virus A/AA/6/60 produced markedly different results from the wild-type A/Alaska/77. The wt virus grew to $10^{6.0}$ and $10^{2.5}$ $EBID_{50}$ in the turbinates and lungs respectively at 3 days post-inoculation, whereas the ca parent grew to a lower titre in the turbinates ($10^{4.3}$ EID_{50}) and was undetectable in the lungs on the same day. In addition the wt virus induced fever and coryza in both infected animals, whereas neither of these conditions arose following infection with a ca parent. The effects produced by the four reassortants fell broadly between those of the parents. The "triple-gene" reassortant, T_6 6-1-1, was attenuated to a similar degree as the ca parent in ferrets. This virus induced no fever and induced coryza in only one of the two animals. Virus was shed for only three days post-infection and was also undetectable in the lungs at this time. The "double-gene" reassortant, T_3 25-1-1 was intermediate in virulence between the wt and ca parents. This virus grew to a higher titre in the turbinates than A/AA/6/60

Table 1. *Growth of parent and recombinant virus strains in Ferrets, and clinical response to infection*

Virus	Dose Administered EID/ml	No. of Ferrets	Log_{10} Titre in indicated Organs T Day 3	T Day 8	L Day 3	L Day 8	Average Duration of Virus Shedding (Days)	No. of Ferrets with Fever	Range of Fever	Average Duration of Fever in days	No. of Ferrets with Coryza
T_4 8-7-1-1	8.3	2	5.5	<1.0	2.5	<1.0	5	2/2	104-5	3	2/2
T_4 31-1-1	8.3	2	5.3	<1.0	2.0	<1.0	4	2/2	104-6	3	2/2
T_3 25-1-1	8.7	2	6.0	<1.0	<1.0	<1.0	4	2/2	104-5	1	2/2
T_6 6-1-1	8.7	2	5.3	<1.0	1.0	<1.0	3	0/2	NA	NA	1/2
A/Alaska/6/77 wild-type	7.5	2	6.0	<1.0	2.5	<1.0	5	2/2	105-6	3	2/2
A/AA/6/60 ca-parent	8.3	2	4.3	<1.0	<1.0	<1.0	3	0/2	NA	NA	0/2

A comparison of the growth rates and clinical effects in Ferrets of viruses exhibiting phenotypic differences in cold-adaptability and temperature-sensitivity *in vitro*. Virus titres are expressed as log_{10} $EBID_{50}$/ml and the level of fever in degrees Farenheit. The reassortant strains T_4 8-7-1-1, T_4 31-1-1 and T_3 25-1-1 are all cold-adapted (ca) and non-temperature sensitive (non-ts) in MDCK cells. Strain T_6 6-1-1 is ca and ts

($10^{6.0}$ EID_{50}/ml) but remained undetectable in the lungs and produced a fever of only 24 hours duration. Both "single-gene" reassortants produced fever of three days duration and grew to detectable titres in the lungs, T_4 8-7-1-1 to $10^{2.5}$ and T_4 31-1-1 to $10^{2.0}$ EID_{50}/ml.

Virus Effect on Ciliary Activity of Hamster Tracheal Rings

Fig. 1 shows the effects of both parent and reassortant viruses on the ciliary activity of hamster tracheal organ cultures. The wt parent, A/Alaska/6/77, reduced ciliary activity at 9 days post-inoculation to 30 per cent of its original value, whereas after a similar incubation period A/AA/6/60 infected cultures retained 75 per cent of original activity.

The two "single-gene" reassortants had similar effects on ciliary activity to the wt parent. T_4 31-1-1 and T_4 8-7-1-1 had reduced activity to 33 and 17 per cent of original values after 9 days. In contrast the "double-gene" reassortant T_3 25-1-1, and the "triple-gene" reassortant T_6 6-1-1, produced similar effects to the ca parent, reducing activity to only 83 and 72 per cent respectively, after a 9 day incubation.

Growth of Viruses in Hamster Lungs and Turbinates

Virus was detected in the lungs and turbinates of hamsters infected with each of the six strains of virus tested. However, the extent of replication in both tissues varied between strains (Table 2). The wild-type parent, A/Alaska/77, grew to high titre in hamster lungs ($10^{4.6}$ $EBID_{50}$/ml) and

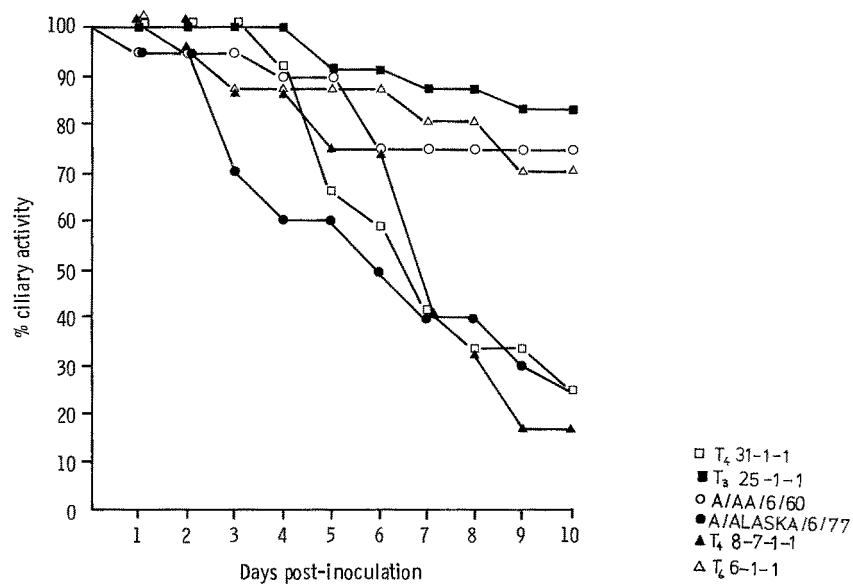

Fig. 1. The effects of parent and reassortant viruses on ciliary activity in Hamster tracheal rings. Organ cultures infected on day 0 with 10^5 $EBID_{50}$ of (○) A/Ann Arbor/6/60, (●) A/Alaska/6/77, (■) T_3 25-1-1, (△) T_6 6-1-1, (□) T_4 31-1-1, and (▲) T_4 8-7-1-1

Table 2. *Growth of parent and reassortant, viruses in the lungs and turbinates of Hamsters*

	Mean Log_{10} $EBID_{50}$/ml (S.D.)					
	Days Post-Inoculation					
	Lungs			Turbinates		
Virus	1	3	4	1	3	4
A/Alaska/6/77	4.0 (0.4)	4.6 (0.4)	3.6 (0.3)	2.1 (0.2)	4.9 (0.5)	1.5 (0.3)
A/Ann Arbor/60	2.0 (0.4)	2.6 (0.2)	1.6 (0.2)	2.6 (0.1)	< 1.0	< 1.0
T_6 6-1-1	1.8 (0.9)	2.2 (0.2)	< 1.0	1.1 (0.8)	< 1.0	< 1.0
T_3 31-1-1	4.0 (0.5)	3.6 (0.3)	2.0 (0.2)	< 1.0	4.2 (1.5)	1.7 (0.8)
T_4 8-7-1-1	3.3 (0.3)	3.0 (0.2)	3.0 (0.1)	2.3 (1.2)	2.6 (0.6)	3.7 (0.9)
T_3 25-1-1	2.8 (0.2)	1.9 (0.4)	< 1.0	2.3 (0.3)	1.2 (0.4)	< 1.0

also grew to a high titre of $10^{4.9}$ $EBID_{50}$/ml in turbinates. As expected the growth of the ca parent was lower in both lungs and turbinates ($10^{2.6}$ and $10^{2.5}$ $EBID_{50}$/ml respectively).

Of the reassortants the "triple-gene", T_6 6-1-1, grew only to low titres of $10^{2.2}$ $EBID_{50}$/ml in lungs and $10^{1.1}$ $EBID_{50}$/ml in turbinates. The "double-gene" reassortant, T_3 25-1-1 grew to higher titres of $10^{2.8}$ $EBID_{50}$/ml (lungs) and $10^{2.3}$ $EBID_{50}$/ml (turbinates). The two "single-gene" reassortants, T_4 31-1-1 and T_4 8-7-1-1 grew to the greatest extent, producing titres of $10^{4.0}$ and $10^{3.3}$ $EBID_{50}$/ml respectively in the lungs and $10^{4.2}$ and $10^{3.7}$ $EBID_{50}$/ml respectively in the turbinates.

Discussion

Cold-adapted reassortant viruses have been extensively studied as possible candidates for live attenuated influenza virus vaccines (1, 2, 18). Despite the large volume of work performed, the genetic basis for determining virulence in these viruses is still largely unsolved (3, 5). One of the major reasons for this has been that reassortants produced in primary chick kidney (PCK) cells showed very little variation in genetic composition (8). By performing reassortment in MDCK cells, several strains containing a far greater number of genes from the wt parent have been produced (14). In this study four of these viruses were assessed for their virulence in previously tested models (7, 10).

The results produced in the three systems were largely consistent. Thus, the two reassortants which derived only a single gene from the ca parent, A/Ann Arbor/6/60, severely reduced ciliary activity in hamster tracheal organ cultures and grew to high titres in hamster and ferret lungs causing fever and coryza in the latter animals. Of the two other reassortants tested, the "double-gene" reassortant, T_3 25-1-1, was more virulent *in vivo*, although there was no detectable difference between this virus and the "triple-gene" reassortant, T_6 6-1-1, in hamster tracheal rings. The results produced in these three tests are in contrast to those found in mice (15). In that study the

"double-gene" reassortant, T_3 25-1-1, and the "single-gene" reassortant T_4 31-1-1 were found to be virulent, whereas the "triple-gene" reassortant, T_6 6-1-1 was attenuated. The viruses were thus divided according to temperature sensitivity in MDCK cells with only the ts virus being attenuated. Interestingly, when the viruses were tested in PCK cells, the two "single-gene" reassortants remained ca and non-ts, while the "double-gene" reassortant became ca and ts (data not shown), indicating that there is also a host factor involved in the expression of temperature sensitivity. Therefore the noted attenuation of the "double-gene" recombinant in these tests may have resulted from the expression in these two animals of a host-variable ts character on both the NA and RNA 2 genes.

Host variable temperature sensitivity has been noted before; when an earlier PCK produced reassortant exhibited a reversion of the ts marker in MDCK cells, temperature sensitivity was retained in the PCK cells, and there was no reversion to virulence in volunteers or in ferrets (8, 18).

A second mechanism which may be acting in concert with the ts hypothesis above is a host-range phenomenon. A/AA/6/60 has been extensively adapted to the avian system by multiple passages in eggs. Thus it grows less well in a mammalian host than a fresh human isolate. This adaptation may be the cause of the lower growth of A/AA/6/60 in turbinates from that of the wt A/Alaska/6/77 virus. Loss of virulence in a reassortant may therefore be due to restrictive host factors together with the ts phenotype.

A/AA/6/60 reassortant viruses which contain only the glycoproteins from the wt parent have been shown to express a consistently stable attenuated phenotype (10). Hopefully, future work utilising "single gene" reassortants, prepared in this way, will demonstrate which genes are involved in attenuation, which genes are essential for attenuation, and how the combination of these genes can result in stable, attenuated strains, suitable for use as live vaccines in man.

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