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Cold-Adapted Reassortants of Influenza A Virus in MDCK Cells

II. Role of the Temperature-Sensitive Property of Cold-Adapted Reassortants in Mice

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Attenuation of influenza viruses for the development of live vaccines can be accomplished by transferring attenuated genes from an attenuated donor virus to the new epidemic or pandemic strain by genetic reassortment (4). One donor virus, an influenza A/Ann Arbor/6/60 cold-adapted variant, was produced by serial passage in primary chick kidney (PCK) cells by gradually lowering the incubation temperature to 25 C which restricts the growth of wild type influenza viruses (1). During adaptation at the lower temperature, the A/Ann Arbor/6/60 virus acquired two phenotypic mutations, a cold-adapted (ca) property and a temperature-sensitive (ts) property at a shutoff temperature of 37–38 C (10). In studies of reassortant viruses derived from the A/Ann Arbor/6/60 donor strain, reassortants possessing the ca and ts properties lost their virulence for animals and humans (2, 3). However, A/Ann Arbor/6/60 donor virus genes which confer both the ca and ts properties and attenuation have not been unequivocally identified since most of the reassortants available for examination have multiple genes from the A/Ann Arbor/6/60 virus and possess both ca and ts properties as a linked phenotype.

Recently, we isolated some limited gene reassortants of A/Ann Arbor/6/60 × A/Alaska/6/77 viruses and demostrated that the ca property can be segregated from the ts property at either 25 or 33 C, which is a permissive temperature for growth of influenza virus (7). Correlations between the reassortants' gene constellations and their phenotypic markers, ca and ts, were also assessed.

The present study explores the functions of both the ca and ts properties in the ca reassortants with respect to their virulence or attenuation in mice.

Influenza A viruses used in this study were the A/Ann Arbor/6/60 (H2N2) ca variant as an attenuated donor strain for mice, the A/Alaska/6/77 (H3N2) wild type, and four ca reassortant viruses. The reassortants were produced in Madin-Darby canine kidney (MDCK) cells by mixed infection with the A/Ann Arbor/6/60 ca donor and the A/Alaska/6/77 viruses at either 25 or 33 C (7).

Table 1 presents a summary of the gene constellations of the ca reassortants and their biological phenotypes as expressed in MDCK cells. Two ($T_6$6-1-1 and $T_6$6-2-1) of the four reassortants exhibited ca and ts phenotypes while the others ($T_3$25-1-1

and $T_4$31-1-1) exhibited ca and non ts phenotypes. Four-week-old CD (ICR) mice obtained from Charles River Breeding Laboratories, Inc. were used as a laboratory model to assess virulence of the reassortants based on their viral

Table 1. Gene constellations and biological properties of ca reassortant viruses

Clone	RNA segments								Biological[a] phenotype
	(Polymerase)			(HA)	(NA)	(NP)	(MP)	(NS)	
	1	2	3	4	5	6	7	8	
$T_3$25-1-1	wt[b]	AA[c]	wt	wt	AA	wt	wt	wt	ca and non-ts
$T_4$31-1-1	wt	AA	wt	wt	wt	wt	wt	wt	ca and non-ts
$T_6$6-1-1	wt	AA	AA	wt	AA	wt	wt	wt	ca and ts
$T_6$6-2-1	wt	AA	AA	wt	AA	wt	wt	wt	ca and ts

[a] ca indicates substantial growth at 25 C; ts indicates little or no growth at 39 C, non-ts indicates substantial growth at 39 C.

[b] wt, RNA derived from A/Alaska/6/77 ($H3N2_{77}$) wild type virus.

[c] AA, RNA derived from A/Ann Arbor/6/60 ($H2N2_{60}$) cold-adapted variant.

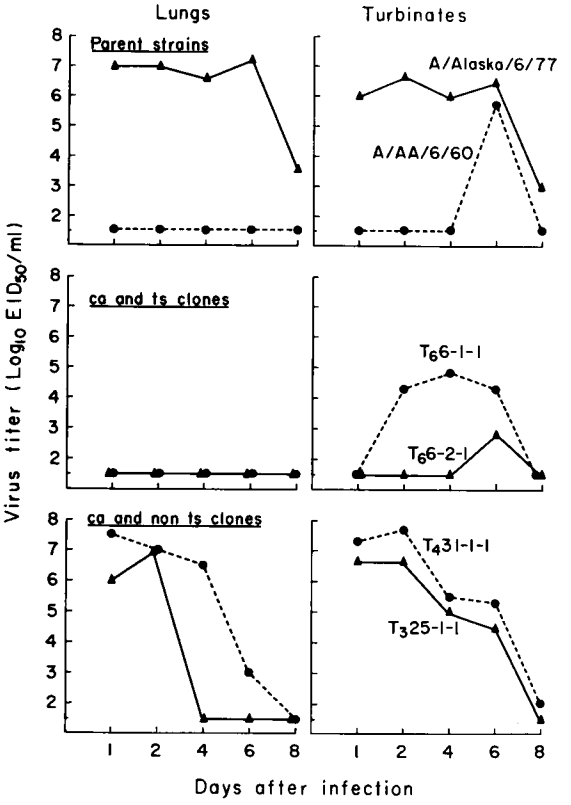

Fig. 1. Growth patterns of ca reassortant viruses and their parental strains in mice.

replicative ability in the lungs and nasal turbinates. The virus dilutions were prepared in a nutrient broth containing penicillin (200 μg/ml) and streptomycin (4 μg/ml) with a dose of 5.0×10^5 PFU/ml at a volume of 5 ml and administered by aerosol exposure for 30 min. Groups of three mice each were sacrificed by cervical dislocation on days 1, 2, 4, 6, and 8 after infection and the lungs and turbinates were removed. The lungs and turbinates were pooled separately, and 10% weight/volume tissue homogenates were prepared as described previously (11). The viral content of the tissue homogenates was evaluated by inoculation into 10-day-old eggs, and incubated for 72 hr at 33 C. The titer, expressed as 50% egg infectious dose (EID_{50}/ml), was calculated by the method of Reed and Muench (8).

Figure 1 shows the virus growth patterns of ca reassortant clones and their parental strains in mouse lungs and turbinates. The wild type A/Alaska/6/77 virus began to replicate in the lungs and turbinates from day 1 after infection. No significant differences were found in the titers of the virus recovered from the lungs (maximum titer: 7.2 $\log_{10}$ EID_{50}/ml) and the turbinates (6.7 $\log_{10}$ EID_{50}/ml). Replication of the A/Ann Arbor/6/60 donor strain was completely restricted in the lungs. The donor strain grew only in the turbinates and a significant titer was observed 6 days after infection, but the maximum titer was 10-fold less than that of A/Alaska/6/77 wild type strain (top panel). Clones $T_6$6-1-1 and $T_6$6-2-1, which possess the ca and ts properties, replicated in the turbinates with maximum titers of 4.8 $\log_{10}EID_{50}$/ml and 2.7 $\log_{10}EID_{50}$/ml, respectively. The growth of these clones in the lungs was also completely restricted (middle panel). On the other hand, two reassortants $T_3$25-1-1 and $T_4$31-1-1, which possess only the ca property, replicated in both the lungs and turbinates with maximum titers comparable to that of A/Alaska/6/77 wild type virus (bottom panel). These results, therefore, indicate that replication of the ca reassortants in the lungs is affected by the acquisition of the ts property from the A/Ann Arbor/6/60 donor virus.

Studies of ts reassortant viruses derived from A/Udorn/72-ts-1A2 donor virus previously demonstrated that the ts genes of the donor virus are responsible for attenuation in humans (6) and that the shutoff temperatures of plaque formation *in vitro* is related to the level of the viral replication in the lungs of hamsters (5). The function of the ts property in attenuation of the A/Ann Arbor/6/60 donor virus is still obscure, however, since the ca reassortants bearing different shutoff temperatures do not show a significant variation in their level of attenuation for humans (9). Although no ca reassortant virus possessing non ca and ts phenotypes has been isolated yet, our present data suggest that the ts property of A/Ann Arbor/6/60 virus is essential in attenuating ca reassortant viruses.

The role of the ca property plays in attenuation of the A/Ann Arbor/6/60 donor virus is also unclear. Two ca and non ts reassortants replicated well in mouse lungs, however, the duration of viral growth was significantly shorter than that of A/Alaska/6/77 wild type virus (Fig. 1 top and bottom panels). A "single gene" reassortant ($T_4$31-1-1) bearing only RNA 2 from the A/Ann Arbor/6/60 virus with all remaining genes from the wild type virus (Table 1) showed significant titers in the lungs for 4 days. $T_3$25-1-1 is a "double gene" reassortant bearing both RNA 2 and neuramini-

dase genes which function individually to express the ca phenotype in MDCK cells and the virus grew for 2 days only. These results strikingly reveal that the shortened viral replication time in mouse lungs is related to the increasing number of ca genes transferred from the A/Ann Arbor/6/60 virus to the ca reassortants.

Recently we obtained similar results in a study using ferrets (submitted for publication). Thus, these findings imply that the ca property of the A/Ann Arbor/6/60 donor virus plays a secondary or supporting role to the ts property in alteration of virulence of the ca reassortant viruses.

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