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Attenuation of Wild-Type Human Influenza A Virus by Acquisition of the PA Polymerase and Matrix Protein Genes of Influenza A/Ann Arbor/6/60 Cold-Adapted Donor Virus

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Wild-type influenza A viruses can be attenuated for humans by the acquisition of genes from the A/Ann Arbor/6/60 cold-adapted (*ca*) donor virus. Six-gene reassortants, that is, viruses containing the hemagglutinin and neuraminidase surface glycoprotein genes of the wild-type virus and the six remaining RNA segments of the *ca* donor virus, are consistently attenuated for humans. During the production of a six-gene reassortant virus containing the surface glycoproteins of the A/Washington/897/80 (H3N2) wild-type virus, a reassortant virus was isolated that contained RNA segments 3 (coding for the polymerase PA protein) and 7 (coding for matrix [M] proteins) from the *ca* parent and all other genes from the wild-type virus. This reassortant virus is referred to as a two-gene reassortant. Because the gene or set of genes responsible for the attenuation of *ca* reassortant viruses has not been defined, we evaluated the two-gene reassortant for level of replication and level of virulence in ferrets and in humans, and we compared its characteristics to those of a six-gene reassortant virus derived from the same two parents. The two-gene reassortant virus infected each of 14 adult seronegative (serum hemagglutination inhibition titer of ≦1:8) volunteers when administered intranasally at a dose of 10^7 50% tissue culture infectious doses, yet it did not produce illness. The level of replication of the two-gene reassortant virus in the upper respiratory tract was equivalent to that of the six-gene reassortant virus. This demonstrates that transfer of the A/Ann Arbor/6/60 *ca* PA polymerase and M genes is sufficient to confer the attenuation phenotype on wild-type influenza A viruses. In the context of previous observations, these results suggest that the A/Ann Arbor/6/60 *ca* donor virus PA polymerase gene plays a major role in the attenuation of *ca* reassortant viruses.

The influenza A/Ann Arbor/6/60 (H2N2) cold-adapted (*ca*) virus has been evaluated extensively as a donor of attenuating genes to newly emerging wild-type influenza A viruses as part of our program for the development of a live attenuated virus vaccine. Six-gene reassortants, that is, viruses containing the hemagglutinin (HA) and neuraminidase (NA) surface glycoprotein genes of the wild-type virus and the six remaining RNA segments of the *ca* donor virus, are consistently attenuated for susceptible humans (2, 15, 22). In addition, these six-gene reassortant viruses appear to be phenotypically stable after replication in vivo (4, 8), even in fully susceptible young children (7, 22, 28).

We have been interested in identifying the gene or genes of the *ca* donor virus which are involved in the attenuation of *ca* reassortant viruses for humans for two reasons. First, it would allow us to determine the number of genes involved in attenuation. This is important since it is reasonable to predict that the greater the number of genes involved in attenuation, the lower the probability that a vaccine virus will revert to virulence. Second, it would direct our attention to the genes that specify attenuation and allow us to search for and identify alterations in these genes that might occur after replication of *ca* reassortant viruses in humans.

Each of the *ca* influenza reassortant viruses evaluated in humans has been found to be attenuated. Studies of reassortant viruses which received only five of the six internal genes from the A/Ann Arbor/6/60 *ca* parent virus have provided some information about the contribution to the attenuation phenotype of individual genes of the *ca* parent (1, 3, 10, 14, 18). Five-gene reassortant viruses which contained a single wild-type internal gene—RNA segment 2 (polymerase PB1), 7 (the matrix [M] gene), or 8 (the nonstructural protein [NS] gene)—were as attenuated for humans as six-gene *ca* reassortant viruses. Thus, each of these RNA segments of the A/Ann Arbor/6/60 *ca* parent is individually not essential for the transfer of the attenuation phenotype. Conversely, in each of the *ca* reassortant viruses which were found to be attenuated, RNA segments 1 and 3 (polymerases PB2 and PA, respectively) and 6 (the nucleoprotein [NP] gene) were derived from the *ca* donor virus. Thus it appears that one or more of these A/Ann Arbor/6/60 *ca* genes are of major importance in specifying attenuation.

During the production of a six-gene reassortant virus containing the surface glycoproteins of the A/Washington/897/80 (H3N2) wild-type virus, a reassortant virus containing RNA segments 3 and 7 (polymerase PA and M genes) from the *ca* parent and all other genes from the wild-type virus (i.e., a two-gene reassortant) was isolated. Because this reassortant virus contained an A/Ann Arbor/6/60 *ca* polymerase PA (thought to be important in the attenuation of *ca* reassortant viruses) in association with only one other gene derived from the *ca* donor virus, we evaluated its virulence in ferrets and humans. The resulting observations were compared with those obtained previously during a study of a six-gene *ca* reassortant virus containing

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RNA SEGMENT	GENE PRODUCT	PARENTAL ORIGIN OF GENES IN COLD ADAPTED INFLUENZA A REASSORTANT VIRUSES									
		A/SCOTLAND/74	A/VICTORIA/75		A/ALASKA/77				A/WASHINGTON/80		
		CR 18	CR 19	WILD TYPE	CR 31-3	CR 31-10	CR 29-2	WILD TYPE	CR 48-2	CR 48-4	WILD TYPE
1	PB2	■	■	□	■	■	■	□	□	■	□
2	PB1	■	□	□	■	■	□	□	□	■	□
3	PA	■	■	□	■	■	■	□	■	■	□
4	HA	□	□	□	□	□	□	□	□	□	□
5	NA	□	□	□	□	□	□	□	□	□	□
6	NP	■	■	□	■	■	■	□	□	■	□
7	M	■	■	□	□	■	■	□	■	■	□
8	NS	□	■	□	■	□	■	□	□	■	□
MEAN PEAK TITER ($LOG_{10}TCID_{50}/ML$)		0.9	0.7	3.4	2.1	1.0	1.5	4.5	0.6	0.6	3.6
% INFECTED		80	92	100	100	94	75	100	100	81	96

■ = GENE DERIVED FROM A/ANN ARBOR/6/60 COLD-ADAPTED VIRUS
□ = GENE DERIVED FROM WILD TYPE VIRUS

FIG. 1. Genotypes of influenza A reassortant viruses derived from A/Ann Arbor/6/60 *ca* × wild-type virus which have been evaluated in humans. Infectivity data are from previously reported trials (3, 13). A 100-fold reduction in level of replication in nasopharynx (mean peak titer) compared with wild type correlates with clinical attenuation.

the same surface antigens in an attempt to gain better understanding of the genetic basis for attenuation of *ca* reassortant viruses (2).

MATERIALS AND METHODS

Virus. The influenza A/Washington/897/80 (H3N2) *ca* reassortant (CR48 clone 2) virus used in this study was produced by mating the influenza A/Ann Arbor/6/60 (H2N2) *ca* donor virus with the cloned influenza A/Washington/897/80 (H3N2) wild-type virus. The passage history of the wild-type virus (antigenically similar to A/Bangkok/1/79) has been published (19). The *ca* reassortant virus suspension administered to humans was produced by Louis Potash (Flow Laboratories, McLean, Va.) and tested for the presence of adventitious agents as previously described (6, 13). The infectivity titer of the viral suspension used in this study (lot E-185) was $10^{7.3}$ 50% tissue culture infectious doses ($TCID_{50}$) per ml. Data from previously reported studies (2, 16) with the influenza A/Washington/897/80 wild-type virus and the influenza A/Washington/897/80 six-gene *ca* reassortant (CR48 clone 4) are presented for the purpose of comparison.

Determination of efficiency of plaque formation at permissive and restrictive temperatures. The efficiency of plaque formation of each virus was determined in primary chicken kidney cell (PCKC) tissue monolayers at 25, 33, or 39°C as described previously (8, 23) and in Madin-Darby canine kidney (MDCK) cell culture at 33, 37, 38, 39, or 40°C (19).

Gel electrophoresis. The parental origin of the genes in the reassortant virus was determined by comparing the migration of the eight RNA segments of the reassortant with those of the two parent viruses in gel electrophoresis as previously described (9, 24). A difference in the migration of the polymerase genes of the A/Washington/897/80 and A/Ann Arbor/6/60 *ca* reassortant was apparent on 1.5% agarose gels in Tris-borate-EDTA buffer plus 0.15% sodium dodecyl sulfate. Electrophoresis was carried out at 37°C for 16 h at 70 V constant voltage. Differences in the remaining five genes were seen with 3.0% polyacrylamide–0.6% agarose mixed gels in Tris-borate-EDTA buffer plus 1.0% sodium dodecyl sulfate. In this case electrophoresis was at 37°C for 16 h at 230 V constant voltage.

The genotypes and attenuation phenotypes of *ca* reassortant viruses derived from the A/Alaska/6/77 (H3N2) and A/Victoria/3/75 (H3N2) wild-type viruses have previously been published (14, 18). An error in the genotype of one of the A/Alaska/6/77 reassortant viruses (CR29-2) was detected with the gel electrophoresis system described above and was confirmed with a different gel electrophoresis system (11). The corrected genotypes along with a summary of the level of replication of the *ca* reassortant viruses are presented in Fig. 1.

Determination of the nucleotide sequence of influenza virus polymerase and M genes. Synthetic oligonucleotide primers were extended by reverse transcription with dideoxynucleotides (26) by using the influenza virus genome RNA as a template. Synthetic oligonucleotide primers whose sequences are specific for each of the three influenza A polymerase genes and a primer specific for the influenza A matrix protein gene were provided by Clayton Naeve (St. Jude's Children's Research Hospital, Memphis, Tenn.). Dideoxynucleotide chain termination sequencing reactions and gels were as described by Naeve et al. (23).

Hybridization of influenza A polymerase genes with radiolabeled DNA probes. Viral RNA was extracted and electrophoretically separated as described above. The RNA was transferred to diazobenzylmethoxyl paper which had been prepared according to the manufacturer's specifications (Schleicher & Schuell Co., Keene, N.H.). Probes consisting of synthetic oligonucleotide primers extended by reverse transcription as described above were allowed to hybridize with the viral RNA bound to the diazobenzylmethoxyl paper. The conditions of hybridization and subsequent washes are described in the legend to Fig. 3.

Ferret studies. The two-gene *ca* reassortant virus and the two parent viruses were evaluated in ferrets as described previously (10). Briefly, groups of four ferrets were anesthetized and inoculated intranasally with a 1-ml suspension containing the quantity of virus indicated in Table 2. In each group, two ferrets were sacrificed on days 3 and 8 after inoculation, 10% (wt/vol) suspensions of the nasal turbinates

and lungs of each ferret were prepared, and the virus titers were determined and expressed in terms of 50% egg infectious doses per milligram of tissue.

Clinical studies. The study protocols were approved by the Clinical Research Subpanel of the National Institute of Allergy and Infectious Diseases and the Human Volunteer Research Committee at the University of Maryland School of Medicine. Healthy adults between the ages of 18 and 35 years who took no medications, had no history of influenza vaccination, and had a serum hemagglutination inhibition antibody titer of 1:8 or less were recruited from among community members in Maryland. Each volunteer gave written informed consent. A description of the isolation facility and the clinical study protocols was published previously (16). Volunteers received 10^6 or 10^7 $TCID_{50}$ of the two-gene *ca* reassortant virus intranasally in a 0.5-ml inoculum. They were housed on the isolation ward at the Center for Vaccine Development, University of Maryland Hospital, where they were observed daily for at least 7 consecutive days after inoculation.

A volunteer was considered ill if symptoms or physical findings consistent with influenza developed. The following criteria were used to categorize illnesses: (i) febrile illness—oral temperature >37.8°C confirmed within 5 min, (ii) systemic illness—occurrence of myalgias alone or with chills or sweats; (iii) upper respiratory tract illness—presence of rhinorrhea or pharyngitis or both on two or more consecutive days; (iv) lower respiratory tract illness—presence of a persistent cough on two or more consecutive days. Diagnosis of systemic and upper and lower respiratory tract illness required the concurrence of two independent examiners. Illness attributed to influenza A virus required confirmation of influenza A infection by laboratory tests (see below).

The amount of serum immunoglobulin A (IgA) and IgG and nasal wash IgA antibody to the purified hemagglutinin of A/Bangkok/1/79 (H3N2) was determined by an enzyme-linked immunosorbent assay as previously described (20, 21, 27). Serum neuraminidase inhibiting antibody levels were determined as described (16) using a reassortant virus which contained the HA of influenza A/equine-1 (H7) and of the NA influenza A/Bangkok/79 (N2).

Nasal wash specimens for virologic study were collected before viral inoculation and daily for 7 to 10 days after inoculation. The method used for virus isolation has been described (16). Isolation of virus from nasal wash specimens or a fourfold rise in antibody titer was considered evidence for infection. Data from all infected volunteers were used in the calculations of mean duration of shedding and mean peak titer of virus shedding.

RESULTS

Determination of the genotype of the influenza A *ca* reassortant virus CR48 clone 2. The influenza A *ca* reassortant virus CR48 clone 2 was shown to contain RNA segments 3 and 7 from the A/Ann Arbor/6/60 *ca* virus, and all other genes were derived from the A/Washington/897/80 wild-type parent (Fig. 2). Two previously studied *ca* reassortant viruses also derived their RNA segment 2 from the respective wild-type parents and RNA segment 3 from the *ca* parent. However, the order of migration of polymerase genes can vary under different gel electrophoresis conditions. Thus the second gel band representing a polymerase gene under one set of electrophoresis conditions may be PB1, whereas under another set of conditions PA may appear as the second band. Therefore gel electrophoresis of the two-gene reassortant virus and of the A/Victoria/75 *ca* and the A/Alaska/77 *ca*

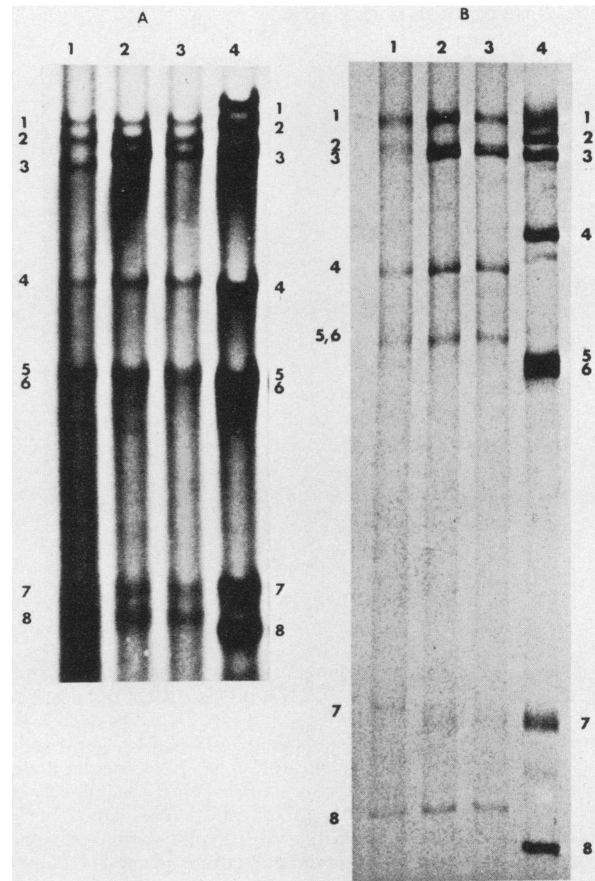

FIG. 2. Genotype of A/Washington/897/80 × A/Ann Arbor/6/60 *ca* reassortant viruses (A) A 1.5% agarose gel in Tris-borate-EDTA buffer plus 0.15% sodium dodecyl sulfate. The samples were electrophoresed at 37°C for 16 h at 70 V constant voltage. (B) A 3.0% polyacrylamide–0.6% agarose mixed gel in Tris-borate-EDTA buffer plus 1.0% sodium dodecyl sulfate. The samples were electrophoresed at 37°C for 16 h at 230 V constant voltage. Lanes for both gels: 1, A/Washington/897/80 wild type; 2, A/Washington/897/80 two-gene *ca*; 3, ferret reisolate of A/Washington/897/80 two-gene *ca*; 4, A/Ann Arbor/6/60 *ca*.

(CR29-2) viruses performed under identical conditions confirmed that each reassortant received the RNA 3 as defined in the conditions described in the legend to Fig. 2A from the A/Ann Arbor/6/60 *ca* donor virus.

To determine the coding assignments of RNA 1, 2, and 3 of the A/Ann Arbor/6/60 *ca* donor virus, hybridization of viral RNA with radiolabeled DNA probes whose sequences were specific for each of the influenza A polymerase genes was performed. This procedure demonstrated that the RNA segment 3 of the influenza A/Ann Arbor/6/60 *ca* virus encodes the polymerase protein PA (Fig. 3), whereas RNA segments 1 and 2 correspond to the PB2 and PB1 genes, respectively. Nonspecific hybridization of each of the probes was also observed in regions corresponding to the HA and NA and NP genes for reasons that are unknown.

Determination of the efficiency of plaque formation at permissive and restrictive temperatures. *ca* viruses form plaques efficiently at 25°C, a temperature restrictive for wild-type influenza A viruses. Also these viruses are restricted in plaque formation above 38 to 39°C, temperatures permissive for wild-type influenza A viruses. To evaluate the

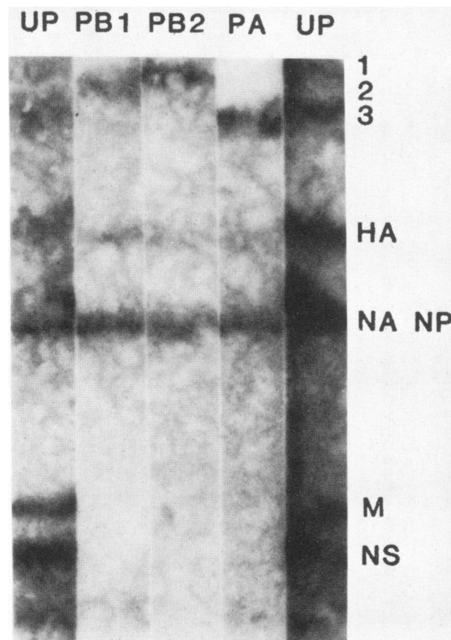

FIG. 3. Hybridization of influenza A/Ann Arbor/6/60 *ca* polymerase genes with radiolabeled DNA probes. Electrophoresis was carried out as described in the legend to Fig. 2A. Hybridization was performed at 42°C in 50% formamide, 0.75 M NaCl, 75 mM sodium citrate, 25 mM sodium phosphate (pH 6.5), 0.2% sodium dodecyl sulfate 0.02% of each bovine serum albumin, ficoll, and polyvirylpyrrolidone, and 100 μg of calf thymus DNA per ml. Washing was at 50°C in 50% formamide, 25 mM sodium phosphate, 0.75 M NaCl, and 75 mM trisodium citrate. Lanes: UP, probe extended from universal primer (consisting of a nucleotide sequence complementary to an initial noncoding RNA sequence contained on every influenza A RNA segment); PB1, PB2, and PA, probe extended from each of the three influenza A polymerase genes.

effect of the transfer of two genes derived from the *ca* donor virus to a wild-type virus on the ability of the resultant reassortant virus to grow at permissive and restrictive temperatures, the A/Washington/897/80 wild type, the A/Washington/897/80 two-gene *ca* reassortant, the A/Washington/897/80 six-gene *ca* reassortant, and the A/Ann Arbor/6/60 *ca* parent were compared for their capacity to plaque in PCKC culture at 25, 33, and 39°C and in MDCK culture at 33, 37, 38, 39, and 40. The two-gene reassortant virus was found to have the *ca* phenotype as indicated by its ability to form plaques on PCKC at 25°C with an efficiency similar to that of the six-gene *ca* reassortant and the A/Ann Arbor/6/60 *ca* parent (Table 1). The A/Washington/897/80 wild-type virus did not form plaques at 25°C; hence the *ca* phenotype was transferred into the reassortant with the two *ca* genes. These results show that the polymerase PA and/or M genes of the A/Ann Arbor/60 *ca* parent virus are sufficient to confer the *ca* phenotype on a wild-type influenza virus.

In PCKC, the two-gene *ca* reassortant virus was found to have the same temperature sensitivity as the six-gene *ca* reassortant virus and the *ca* donor virus. An additional study performed in MDCK tissue culture revealed that the shutoff temperature of the two-gene reassortant virus was intermediate between that of the wild-type virus and the six-gene reassortant virus, indicating that in MDCK cells the two A/Ann Arbor/6/60 *ca* genes present in this reassortant did not confer the same level of temperature sensitivity as the six internal genes of the A/Ann Arbor/6/60 *ca* virus.

Level of replication and virulence in ferrets. The two-gene *ca* reassortant virus infected each of four ferrets when inoculated intranasally at a dose of 10^7 50% egg infectious doses (Table 2). The level of virus replication of the two-gene *ca* virus in the nasal turbinates was similar to that of the A/Washington/897/80 six-gene *ca* reassortant virus and the A/Ann Arbor/6/60 *ca* virus. Virus replication was detected in the lungs of ferrets infected with A/Washington/897/80 wild-type virus, but not in the lungs of ferrets infected with the two-gene *ca* or the six-gene *ca* reassortant viruses. Although the two-gene *ca* reassortant virus grew to the same level as the six-gene reassortant, it produced more illness than the six-gene *ca* reassortant, but less than the wild-type parent virus. These differences were not significant due to the small number of ferrets that were studied.

Level of replication and virulence in humans. The two-gene *ca* reassortant virus was evaluated in seronegative adult volunteers at a dose of 10^6 and 10^7 $TCID_{50}$. To confirm that there was no difference in susceptibility to infection among the groups of volunteers who received the two-gene *ca*, six-gene *ca*, and wild-type viruses, preinfection neuraminidase inhibition and nasal wash HA IgA enzyme-linked immunosorbent assay antibody titers were determined. The neuraminidase inhibition titers ($-\log_2$ mean ± standard error) were as follows: two-gene *ca* 10^7 $TCID_{50}$ group, 0.6 ± 0.2; six-gene group, *ca* 0.8 ± 0.3; wild type, 0.9 ± 0.3. The nasal wash HA IgA enzyme-linked immunosorbent assay titers ($\log_2$ mean ± standard error) were as follows: two-gene *ca* 10^7 $TCID_{50}$ group, 3.4 ± 0.6; six-gene group *ca*, 2.3 ± 0.2; wild type, 2.6 ± 0.3. Since volunteers were selected to have hemagglutination inhibition antibody titers of ≦1:8 and were also equivalent in preinfection serum HA IgG ELISA titers (Table 3), we judged the groups to have equal susceptibility to infection with influenza A/Washington/80 (H3N2) subtype viruses. At 10^6 $TCID_{50}$, six of eight volunteers were infected with the two-gene *ca* virus, but none shed virus (data not shown). At a dose of 10^7 $TCID_{50}$, each of the 14 volunteers was infected, and three shed virus (Table 3). Infection of the volunteers who did not shed virus was documented by the

TABLE 1. Characterization of the *ca* and *ts* phenotypes of reassortants of A/Washington/897/80 wild type × A/Ann Arbor/6/60 *ca* viruses

Virus	Log₁₀ reduction in PFU/ml at indicated temp compared with permissive temp (33°C)					
	In PCKC cells		In MDCK cells			
	25°	39°C	37°C	38°C	39°C	40°C
A/Washington/80 wild type	>5.0[a]	0.7	0.1	0.1	0.5	0.7
A/Washington/80 2-gene *ca*	0.6	>4.3[b]	0.3	0.6	1.2	3.1
A/Washington/80 6-gene *ca*	0.7	>4.7	0.1	0.8	>3.6	>3.6
A/Ann Arbor/60 *ca*	0.0	>5.3	0.1	1.7	>4.5	>4.5

[a] The *ca* phenotype is defined as the ability of a virus to efficiently form plaques at 25°C.

[b] The shutoff temperature is defined as the temperature at which the ability of a virus to form plaques is reduced 100-fold compared with that at 33°C. Titrations were performed as previously described on primary CKC cells (4, 16) or on MDCK cells (17).

TABLE 2. Infection of ferrets with reassortants of influenza A/Washington/897/80 (H3N2) and A/Ann Arbor/6/60 cold-adapted (*ca*) viruses

Virus	Log_{10} EID_{50}[a]	No. of ferrets	Virus shedding: Log_{10} titer in indicated tissues[b]: Nasal turbinate Day 3	Nasal turbinate Day 8	Lung Day 3	Lung Day 8	Mean duration[b] of nasal shedding (days)	Illness: % Febrile	% Coryza
A/Washington/80 wild type	6.7	4	5.0[c]	2.0	1.2	<0.5	4	100	100
A/Washington/80 2-gene *ca*	7.0	4	3.5	<0.5	<0.5	<0.5	2	50	50
A/Washington/80 6-gene *ca*	7.5	8	3.2	<0.5	<0.5	<0.5	3	0	13
A/Ann Arbor/60 *ca*	7.5	4	4.5	<0.5	<0.5	<0.5	3	0	0

[a] Anesthetized ferrets received the indicated 50% egg infectious dose of virus intranasally.
[b] Half of the ferrets in each group were sacrificed on the indicated day; 10% (wt/vol) suspensions of nasal turbinate and lung tissues were made and cultured for virus in eggs (four eggs per dilution). Additional ferrets had daily nasal swabs to determine the duration of virus shedding.
[c] Virus titers are expressed in log_{10} 50% egg infectious doses per milligram of tissue.

demonstration of a fourfold rise in serum HA IgA or IgG ELISA antibody titer (or both). Both the magnitude and duration of virus shedding in the volunteers who received the two-gene *ca* reassortant virus were nearly identical to that of the volunteers infected with the A/Washington/897/80 six-gene *ca* reassortant virus. Both of these *ca* reassortant viruses were shed in significantly lower titer and for significantly shorter duration than was the wild-type virus parent. Each of five isolates obtained from nasal washes of volunteers infected with 10^7 $TCID_{50}$ of the two-gene *ca* reassortant virus retained the *ts* and *ca* phenotypes of the virus inoculated.

None of the 20 volunteers who were infected with the two-gene *ca* reassortant virus developed illness during the study, whereas 11 of 24 (45%) of volunteers infected with wild-type virus became ill ($P < 0.02$, Fisher's exact test). The six-gene *ca* virus produced illness with very low frequency (3%).

Analysis of the segregation of the attenuation, *ca*, and *ts* phenotypes with *ca* gene(s). One *ca* reassortant of A/Victoria/75 and three *ca* reassortants of A/Alaska/77 whose genotypes had been previously reported were studied. This analysis indicated that the A/Alaska/77 *ca* reassortant viruses (CR29 clone 2) derived its RNA segment 2 (polymerase PB1) from the wild type parent rather than from the *ca* parent, contrary to previous reports (3, 14, 19). Additionally, the M gene of A/Alaska/77 *ca* reassortant CR31-10 did not comigrate with the M gene of either parent. Nucleotide sequences of a portion of the M genes which encode the M2 proteins of the reassortant CR31-10 and the two parent viruses were determined. The partial sequences of the CR31-10 and the A/Ann Arbor/60 *ca* M genes were identical, whereas there was a difference of 7 of 120 nucleotides between the sequences of the CR31-10 M gene and that of the A/Alaska/77 parent virus (data not shown). It therefore appears that the CR31-10 M gene is a variant which was derived from the *ca* parent as reported earlier. All other gene assignments were consistent with those previously reported (data not shown).

Of the five previously studied five-gene *ca* reassortant viruses, the A/Victoria/75 *ca* and the A/Alaska/77 *ca* reassortant CR29-2 contained wild-type polymerase PB1 genes.

TABLE 3. Immunization of seronegative volunteers with reassortants of influenza A/Washington/897/80 and A/Ann Arbor/6/60 cold-adapted (*ca*) virus

Virus	Log_{10} $TCID_{50}$[a]	No. of vols	% Infected[b]	Virus shedding: % Shedding	Mean duration (days) ± SE[c]	Peak mean titer (log_{10} $TCID_{50}$/ml) ± SE[c]	Antibody response: Serum HA IgG ELISA (reciprocal log_2 titer) ± SE: Preimmunization	Postimmunization	% With antibody titer increase[d]	% With illness[e]
A/Washington/80 wild type	6.0	24	96	84	4.0 ± 0.5	3.6 ± 0.4	10.1 ± 0.2	13.3 ± 0.4	96	45
A/Washington/80 2-gene *ca*	7.0	14	100	21[f]	0.4 ± 0.2[g]	0.6 ± 0.1[g]	9.6 ± 0.4	11.8 ± 0.0	100	0[f]
A/Washington/80 6-gene *ca*	7.0	31	81	19[f]	0.2 ± 0.1[g]	0.6 ± 0.0[g]	10.2 ± 0.3	11.7 ± 0.3	81	3[f]

[a] Seronegative (hemagglutination inhibition titer of ≦1:8) volunteers received 0.5 ml of virus intranasally. Viral genotypes are described in the text and shown in the figures. After inoculation volunteers were examined, and nasal wash specimens were collected daily for 7 (two-gene *ca* virus) or 10 (other groups) days.
[b] Virus recovery or an antibody titer increase signified infection.
[c] Data from each infected volunteer were used for calculations.
[d] A significant increase in antibody titer was documented by a fourfold or greater rise in one or more of the following: serum hemagglutination inhibition or neuraminidase inhibition, serum or nasal wash IgG, or IgA ELISA.
[e] Volunteers were considered ill if they developed any of the following syndromes: fever (oral temperature of >37.8°C), systemic illness (myalgias or chills and sweats), upper respiratory tract illness (rhinitis, pharyngitis, or both observed on 2 consecutive days), or lower respiratory tract illness (persistent cough on 2 or more consecutive days).
[f] $P < 0.02$ by Fisher's exact test compared with group receiving wild-type virus.
[g] $P < 0.001$ by Student's t test compared with group receiving wild-type virus.

The A/Scotland/74 *ca* and the A/Alaska/77 *ca* CR31-10 viruses contained a wild-type NS gene. The M gene was derived from the wild-type virus in another A/Alaska/77 *ca* reassortant virus (CR31-3) (14, 18).

A comparison of the levels of replication in seronegative volunteers of *ca* reassortant viruses with various "internal" gene constellations is presented in Fig. 1. In our experience a 100-fold reduction in the level of replication of a reassortant virus in the human nasopharynx correlates with attenuation. Each of these reassortant viruses deriving five genes from the A/Ann Arbor/6/60 *ca* virus was *ca* and was attenuated in ferrets and in seronegative adult human volunteers. These studies indicated that polymerase PB1 and the M and NS genes of the A/Ann Arbor/6/60 *ca* donor virus are not essential for the attenuation of *ca* reassortant viruses. The current study provides evidence that the *ca* polymerases PB1 and PB2 and the NP and NS genes are not essential for attenuation of *ca* reassortant viruses. Considering these data together, only the *ca* polymerase PA gene cosegregates with the attenuation phenotype. Similarly, only the *ca* polymerase PA gene has been present in all reassortants of the A/Ann Arbor/6/60 *ca* × wild-type viruses which have displayed the *ca* phenotype (5). These data suggest that the polymerase PA gene of the A/Ann Arbor/6/60 *ca* virus plays a major role in the transfer of the *ca* and attenuation phenotypes to wild-type viruses.

DISCUSSION

The current study shows that the transfer of as few as two genes derived from the A/Ann Arbor/6/60 *ca* donor virus is sufficient to confer attenuation and the *ca* phenotype on virulent wild-type influenza A viruses. Also, in the context of previous observations, it appears that the A/Ann Arbor/6/60 *ca* virus PA polymerase plays a major role in transfer of the *ca* and attenuation phenotypes to reassortant viruses. Other genes of the *ca* donor virus do not appear to be essential for the transfer of these two phenotypes. This finding is of interest for several reasons. First, if the *ca* and attenuation phenotypes are transferred by the same A/Ann Arbor/6/60 *ca* gene, it is possible that the *ca* phenotype could be used as an in vitro phenotypic marker whose absence in *ca* reassortant viruses would indicate virulence. This would be especially true if the *ca* and attenuation phenotypes were due to the same genetic lesion. Second, by identifying the specific gene or genes responsible for the attenuation phenotype, the nature of the genetic lesion responsible for attenuation could be more easily characterized. For example, if studies with reassortant viruses were to show that there was only one A/Ann Arbor/6/60 *ca* gene whose transfer was involved in conferring of the attenuation phenotype, sequence analysis of the RNA of this gene would indicate whether the phenotype is due to a potentially stable lesion (such as a deletion mutation) or to a potentially unstable lesion (such as a point mutation). Analysis of the RNA sequence of the segment(s) involved in attenuation derived from virus isolates from experimentally infected volunteers could thus indicate the likelihood of reversion to virulence in vivo.

This study also demonstrates a dissociation between the *ts* phenotype and the *ca* and attenuation phenotypes. The chemically induced *ts* mutants studied in the 1970s showed a correspondence between the degree of temperature sensitivity and the level of attenuation. In contrast, the two-gene *ca* reassortant virus is fully attenuated in adult seronegative volunteers despite a shutoff temperature that is higher than those of two virulent *ts* mutants (12, 17, 25). Thus, the basis of attenuation of reassortants of the A/Ann Arbor/6/60 *ca* donor virus must involve properties other than temperature sensitivity.

Finally, in this study we found that a *ca* reassortant virus which is partially virulent in ferrets may be completely attenuated in humans. Whether this is due to differences in the interactions of host and virus proteins or differences in preexisting immunity between ferrets and seronegative adult humans (who despite hemagglutination inhibition titers of ≦1:8 have experienced previous influenza A infections) is unclear. Until this issue is clarified, the ferret remains a useful model for evaluation of the safety of new *ca* reassortant viruses before clinical trials, but caution must be exercised in attempting to correlate levels of attenuation in ferrets and in humans.

Several questions remain unanswered concerning the genetic basis of attenuation of *ca* reassortant viruses. Among these are whether the polymerase PA gene of the A/Ann Arbor/6/60 *ca* virus is essential for attenuation, whether other gene(s) also can play a role in attenuation, and whether the *ca* phenotype is a sine qua non for attenuation. The genetic basis of the *ts* phenotype also remains unresolved. We plan to address these questions by studying single-gene reassortant viruses derived from a cross between the A/Ann Arbor/6/60 *ca* parent virus and a current wild-type influenza A virus. (Single gene reassortant viruses contain only one internal gene derived from the *ca* parent virus and all other genes derived from the wild-type parent virus.) Study of these viruses may unambiguously allow us to assign biologic properties to specific RNA segments.

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