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DEVELOPMENT OF COLD-ADAPTED RECOMBINANT LIVE, ATTENUATED INFLUENZA A VACCINES IN THE U.S.A. AND U.S.S.R.

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INTRODUCTION

Influenza remains a major public health problem in many countries. Periodic epidemics disrupt economic and social activities within entire communities, impose sudden large demands on available medical facilities, and can cause excess mortality, particularly in the elderly or those with certain chronic illnesses [5,7,21].

Vaccination can provide significant protection against infection and illness during natural epidemics, but this protection is usually considered to be of limited duration, particularly due to the changes in antigenic specificity of influenza virus surface glycoproteins (hemagglutinin (HA) and neuraminidase (NA)) which continually occur [58]. This problem affects the two methods that exist for influenza vaccination. Inactivated (killed) vaccines must be constantly updated to include newer strains, which in any event often induce maximum antibody responses not to themselves but to previously prevalent strains, as expounded in the 'Doctrine of Original Antigenic Sin' [48,62]. Live, attenuated virus vaccines might be expected to produce a more long-lasting protection than inactivated vaccines on the assumption that they would mimic natural infection which, for unknown reasons, probably protects longer than inactivated vaccines [15]. However, live vaccines must be infectious, attenuated, induce protective immunity and be genetically stable, a combination of properties that might not be obtained in a short space of time with each important new strain (antigenic variant) as it appears.

The ability to recombine influenza viruses by mixed infections during which the segmented RNA genome can reassort [52] raised the prospect that once a suitable attenuated vaccine strain of influenza A was prepared, this property could be transferred to a new strain with contemporary HA and NA antigens [8,35,37]. Provided only that these glycoproteins did not contribute significantly to other (non-antigen dependent)

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vaccine-required properties, attenuated recombinants should be rapidly obtained in this way.

This strategy has been adopted now, using several prospective attenuated strains as donors in recombinations with contemporary wild-type (*wt*) viruses. The work with highly laboratory-passaged attenuated strains, such as A/PR/8/34 (H1N1) or A/Okuda/57 (H2N2), has been described elsewhere [10,16,39,47,57]. Although subjected to extensive clinical studies, and in some cases commercially dsitributed, these vaccines are not currently being used except for occasional special research investigations. A second approach to the use of recombinant live virus vaccines was that of Chanock and coworkers, who followed a suggestion by Mackenzie [37] that temperature-sensitive (*ts*)* variants might be attenuated. Methods were developed for introducing one or more single step lesions into influenza viruses and then transferring the *ts* genes to new *wt* strains by recombination. Although the two most widely studied *ts* donors (TS 1-E and TS 1-A-2) had many desirable properties for live vaccine use, instability of the phenotypic *ts* marker (associated in at least one case with reversion to virulence) proved a major obstacle [60].

A third alternative in the preparation of recombinant live virus vaccines is the use of cold-adapted (*ca*) viruses as attenuated donor strains. This concept has been studied in the Soviet Union since at least 1965 [4] where in many years *ca* influenza viruses have been empirically selected for use in widespread vaccination activities. Because of the reported success in the Soviet Union with *ca* live vaccines, research in this area has been undertaken also in the United States, including investigation of methods for obtaining such variants and testing their biological properties in vitro [30], in animal models [32] and in some preliminary human volunteer studies [9]. In both countries there has been active application of genetic recombination (reassortment) and of newly developed molecular techniques, to analyze the properties of donor-attenuated *ca* viruses and their recombinants as an aid to the development of live, attenuated influenza vaccines. These recent advances are the subject of this review.

DONOR-ATTENUATED STRAINS

Method of obtaining donor-attenuated strains

In the U.S.S.R. and the U.S.A. a large number of field strains of influenza A viruses of different subtypes have been adapted to growth at suboptimal temperatures (<30°C) as a potential method for attenuating the virus' virulence [1,4,30,31,34,54]. Only a small number of these strains, however, have been used in recombination experiments designed to transfer the cold-adaptation property to candidate vaccines for human use. In the U.S.S.R. these donor strains consist of A/Leningrad/9/46(H1N1) and A/Leningrad/134/

* *ts* is used to designate viruses exhibiting $>10^3$ $\log_{10}$ reduction in infectious titer at non-permissive temperatures of >38°C compared to titer at permissive temperature of 33–34°C.

57(H2N2) [4], and in the U.S.A. most recombination studies have used A/Ann Arbor/6/60(H2N2) as the *ca* donor parent.

Prior to adaptation to growth at 25°C, the Leningrad viruses received 20 or more chick embryo (egg allantoic cavity) passages at 32°C. Subsequently they were passaged 17–47 times in eggs at 25–26°C for 4 or 5 days. During the cold-adaptation procedure, it was twice necessary to passage the H2N2 virus at 31°C for 2 days to maintain viability and later passages were at 26°C for 4 days rather than 25°C for 5 days, with higher yield obtained [4]. Experience in the U.S.A., starting with viruses that had fewer egg passages at 32°C, was consistent that field strains of influenza did not normally contain *ca* mutants capable of replicating efficiently at 25°C. Accordingly, a procedure of stepwise adaptation to growth at lower temperatures was adopted. A field strain of H2N2 influenza, A/Ann Arbor/6/60, was isolated in primary chicken kidney (CK) cells and then passaged 10 times in CK cells at 33°C and 10 times at 30°C. Although on initial passage at 30°C the virus yield was 2–3 logs lower than at 33°C, after 10 passages at this temperature virus was selected that grew equally efficiently at 30°C as the original field strain had done at 33°C. This process of progressive adaptation was repeated by growing the mutant at 27°C for 10 passages and finally at 25°C for 10 passages (Table 1). It should be noted that this line of *ca* A/Ann Arbor/6/60 differs from that developed for evaluation in an animal model [30] in its lower number of CK passages, and absence of mouse lung passages, prior to adaptation to growth at 25°C.

Biological properties of donor-attenuated strains

The *ca* A/Ann Arbor/6/60 mutant and the Leningrad *ca* mutants selected for high growth at temperatures below 30°C shared the property of restricted replication in embryonated eggs at the elevated temperature of 39–40°C (Table 2). Characterization of replication of the *ca* A/Ann Arbor/6/60 virus has been done not only in embryonated eggs, but also by plaque titration in primary CK cells. No detectable plaques are produced

TABLE 1

Isolation and passage history of cold-adapted influenza A/Ann Arbor/6/60 mutant used for preparing recombinant vaccine candidates

Passage level	Conditions
1	Throat swab, incubate at 36°C in CK cells
2	CK_1 isolate, incubate at 36°C in CK cells
3–9	Serial passages, incubate at 33°C in CK cells
10–16	Serial passages, incubate at 30°C in CK cells
17–23	Serial passages, incubate at 25°C in CK cells
23–29	Serial plaque purification, at 25°C in CK cells
30–32[a]	Serial passage at 25°C in specific pathogen-free hens' eggs

[a] Recombinants prior to AA-CR 22 were prepared from a stock passaged in standard hens' eggs.

TABLE 2

Replication temperature markers of cold-adapted viruses used as parents in the preparation of recombinant vaccine candidates

	Replication of cold-adapted parental virus						
	in eggs[a] at (°C)				in CK cells[b] at (°C)		
	25	28	33	39–40	25	33	39
A/Leningrad/9/46(H1N1)	2.25	8.25	8.25	2.25	ND[c]	ND	ND
A/Leningrad/134/57(H2N2)	4.75	8.25	8.25	2.25	ND	ND	ND
A/Ann Arbor/6/60(H2N2)	7.50	ND	8.50	1.30	8.30	8.70	<3.0

[a] EID_{50} (log_{10}) after 2–3 days at 33°C or 39°C and after 5 days at 25°C.
[b] Plaque-forming units (log_{10}) after 3–4 days at 25°C, 33°C or 39°C, and after 5–7 days at 25°C.
[c] Not done.

at 39°C in contrast to the wild-type (*wt*) virus. Similar results are obtained in Madin–Darby canine kidney (MDCK) cell cultures (not shown). The *ca* mutant, but not the *wt* A/Ann Arbor/6/60 virus, also produces plaques in primary CK cells at 25°C, which provides a convenient mechanism for preparing *ca* recombinants, as described below.

Another biological property analyzed for the *ca* A/Ann Arbor/6/60 virus is its virulence in the ferret animal model system. The *wt* virus replicates to a higher titer than its *ca* derivative and is isolated from lungs as well as turbinates (Table 3). Greater replication of the *wt* virus is accompanied by a febrile response and the production of purulent rhinitis which are not produced by infection with the *ca* mutant.

Detection of genetic mutations in donor ca *virus strains*

Most work has been directed towards determining the genetic basis for the *ts* phenotype of the *ca* viruses. Traditionally, determination of the gene possessing the conditional lethal *ts* lesion in one mutant microorganism has been determined by analyzing the ability of the mutant to complement or recombine with other *ts* mutants whose lesions

TABLE 3

Replication of wild-type and cold-adapted A/Ann Arbor/6/60 virus in ferrets[a]

Time post-infection (h)	Infectivity titer[b]			
	Wild-type		Mutant	
	Turbinates	Lungs	Turbinates	Lungs
48	5.7	1.7	4.3	<1.0
96	5.5	2.5	3.5	<1.0
192	3.5	1.0	<1.0	<1.0

[a] Ferrets were infected intranasally with about $10^{7.5}$ EID_{50} of virus.
[b] Log_{10} EID_{50}/ml of 20% tissue suspension.

have been previously defined. Because influenza viruses have a segmented genome, reassortment of individual RNA segments can occur in mixed infections analogous to recombination, so that both recombination and complementation analyses of *ts* lesions are possible.

Each of the *ca* Leningrad donor viruses has been examined by recombination analysis with a set of six previously characterized fowl plague virus *ts* mutants which have single-step *ts* lesions in genes coding for their internal proteins [6,18–20,24,38]. The A/Leningrad/9/46 (H1N1) virus failed to recombine with fowl plague mutants possessing lesions in the P3 gene (RNA 1), the P1 gene (RNA 2) and the matrix protein gene suggesting the presence of mutations in these three genes in the *ca* Leningrad strain (Table 4). The *ca* A/Leningrad/134/57(H2N2) virus, passaged at 25°C 17 times, failed to recombine with fowl plague virus mutants having lesions in genes coding for P1, nucleoprotein, and matrix protein, also suggesting the existence of mutations in three genes in this Leningrad *ca* virus. After a further 30 passages at 25°C, the A/Leningrad/134/57 *ca* virus additionally lost the ability to recombine with mutants possessing *ts* lesions in the P1 gene (RNA 2) and the non-structural protein gene (RNA 8), so that in this more highly passaged virus a total of five genes may contain *ts* lesions. Due to possible incompatibilities between certain gene constellations that can, for example, prevent the detection of non-*ts* recombinants in some host systems [53], definitive proof of the site of *ts* mutations (as with *ca* A/Ann Arbor/6/60) will require further detailed genetic and molecular analysis.

The *ca* A/Ann Arbor/6/60 mutant has been analyzed by complementation analysis with a series of single-step *ts* mutants prepared by Murphy and colleagues [45,56] and by recombination analysis with single-step *ts* mutants prepared by Sugiura and colleagues [59]. In the former case it was found that the *ca* mutant failed to comple-

TABLE 4

Genetic analyses of cold-adapted (*ca*) Leningrad virus by recombination analysis with mutants of influenza A fowl plague virus (Dobson Weybridge strain) having *ts* lesions in single genes

FPV mutants		Recombination frequency with *ca* virus		
Clone	Protein with *ts* mutation	A/Leningrad/9/46(H1N1)	A/Leningrad/134/57(H2N2)	
		25°C x 37[a]	25°C x 17[a]	25°C x 47[a]
29[b]	P3	< 0.01	< 0.01	<0.01
131[b]	P1	< 0.01	22	<0.01
166[b]	P2	1.7	8.0	2.0
US1[c]	NP	1.2	< 0.01	<0.01
3031[b]	M	< 0.01	< 0.01	<0.01
mN3[c]	NS	15	4.0	<0.01

[a] Number of times passaged at 25°C.

[b] Described in refs. 18–20, 24, 38.

[c] Described in ref 1. These mutants were kindly supplied by Dr. B. Mahy.

ment a virus R1 [54], which has a defect in its P3 protein [49], although complementation did occur with *ts* mutants from six other genetic groups. Recombination experiments comparing mutant A/Ann Arbor/6/60 with Sugiura's *ts* mutants of WSN virus, however, showed that the A/Ann Arbor/6/60 mutant could recombine with the WSN mutant having a lesion in its P3 protein, but could not recombine with a WSN mutant having a lesion in its P1 protein [13].

To resolve these contradictory findings, the genome composition was determined for several recombinants derived from the *ca* A/Ann Arbor/6/60 virus. The surprising result was obtained (Table 5) that every gene of mutant A/Ann Arbor/6/60 was present in one or more non-*ts* recombinant viruses. This indicated that possibly no single gene contained a *ts* lesion in the *ca* mutant, but rather a combination of two or more of its genes was necessary to obtain a *ts* phenotype. The non-*ts* recombinant (S34) was found to contain a single *wt* gene (P3, coded by RNA 1), showing that this gene of the mutant A/Ann Arbor/6/60 was one of those involved in the group of genes necessary for a *ts* phenotype. Examination of recombinants having either *ts* or non-*ts* phenotypes indicated that whenever the A/Ann Arbor/6/60 P3 and matrix proteins segments were present in the virus, the virus was *ts*. Thus, it is suggested that the *ts* phenotype of mutant A/Ann Arbor/6/60

TABLE 5

Properties of recombinants derived from mixed infections of A/Ann Arbor/6/60 mutant and WSN mutants or A/Ann Arbor/9/73 wild-type

Recombinant clone designation[a]	Gene derivation[b]								EOP (%)
	P3 (RNA 1)	P1 (RNA 2)	P2 (RNA 3)	HA	NP	NA	M	NS	p.f.u. 39°C/ p.f.u. 33°C
Ia_3	A	A	W	W	A	A	W	A	12
Iu_2	A	W	W	W	W	A	W	W	67
Im_3	A	W	W	W	A	A	W	A	25
IIg_1	A	W	A	A	A	A	W	A	25
Vh_5	W	W	A	W	A	W	W	W	50
$VIIb_1$	W	W	W	A	W	A	A	W	100
$VIIc_1$	W	A	W	W	W	A	A	A	25
S32	O	O	A	A	A	A	A	A	51
S34	O	A	A	A	A	A	A	A	19
S1	A	O	O	O	O	A	A	A	< 0.001
S10	A	A	A	A	A	A	A	O	< 0.002
M4	A	O	A	O	A	O	A	A	< 0.002
No ts^+ recombinants with A/Ann Arbor/6/60 gene:	4/9	3/9	4/9	4/9	6/9	8/9	4/9	6/9	
No *ts* recombinants with A/Ann Arbor/6/60 gene:	3/3	1/3	2/3	1/3	2/3	2/3	3/3	2/3	

[a,b] A indicates the gene derived from the A/Ann Arbor/6/60 mutant; W indicates the gene derived from a WSN mutant; O indicates the gene derived from the A/Ann Arbor/9/73 wild-type virus.

results from synergism between these two viral proteins [13]. These findings do not exclude the possibilities that additional A/Ann Arbor/6/60 genes may affect the overall level of temperature sensitivity, or that the constellation of genes required for temperature sensitivity may vary depending on the *wt* parent used in recombination.

The existence of mutations in all of the genes of *ca* A/Ann Arbor/6/60 virus has been detected by a combination of different biochemical techniques [11]. For example, comparison of RNA oligonucleotide maps indicated the existence of at least five detectable differences between the *wt* and *ca* A/Ann Arbor/6/60 viruses in the approximately 10% of the genome that is analyzed by this procedure (Fig. 1). One spot difference occurred in each of the RNA segments RNA 1 (P3), 3 (P2) and 4 (HA), and two spot differences occurred in the nucleoprotein gene.

The presence of mutations in genes coding for HA, NA, nucleoprotein, matrix protein and non-structural protein was determined by electrophoresis of heteroduplex RNAs prepared between *wt* virion RNA and *ca* virus complementary RNA as described by Hay et al. [23], and existence of a mutation in RNA 2 (P1 gene) has been found by analysis of temperature sensitivity of virion transcriptase activity in recombinants of known genotype [26]. The multi-step nature of mutations in *ca* A/Ann Arbor/6/60 may explain the peculiarities of its reactions in complementation and recombination analyses.

Biochemical studies have failed, however, to uncover any marker which might explain the virus' temperature sensitivity of replication. All detectable polypeptides of the A/Ann Arbor/6/60 *ca* virus are synthesized with the same efficiency at the non-permissive temperature of 39°C as is observed with the *wt* virus when tested in either CK or MDCK cell cultures, although an altered migration rate of polypeptide P2 has been observed [11]. The findings suggest that the mutation responsible for temperature sensitivity affects a late function in viral replication rather than any of the functions occurring prior to transcription of virion RNA into messenger RNAs and their translation into viral polypeptides.

Infectiousness, reactogenicity and immunogenicity of donor-attenuated strains

These properties have been examined for both of the *ca* Leningrad viruses by infection of susceptible children. A total of 5164 children between the ages of 1 and 6 years old have been infected with the H2N2 A/Leningrad/134/57 *ca* virus. The studies were conducted in 1961–1964, years when some children would be expected to have hemagglutinin-specific antibody to the H2N2 virus, which caused the Asian influenza pandemic in 1957, and periodic epidemics thereafter until 1968. Immunization of these healthy children involved intranasal administration with a sprayer of two doses (at about a 2 week interval), each containing approximately 10^7 EID_{50} of the virus. Fever and clinical reactions were monitored for 5 days following immunization, and immunogenicity was evaluated by measuring the frequency of seroconversions detected by hemagglutination-inhibition (HI) tests. The percentage of children reported as showing febrile reactions (temperature >38.5°C) following this vaccination ranges from 0.1% to 0.8% with 92%

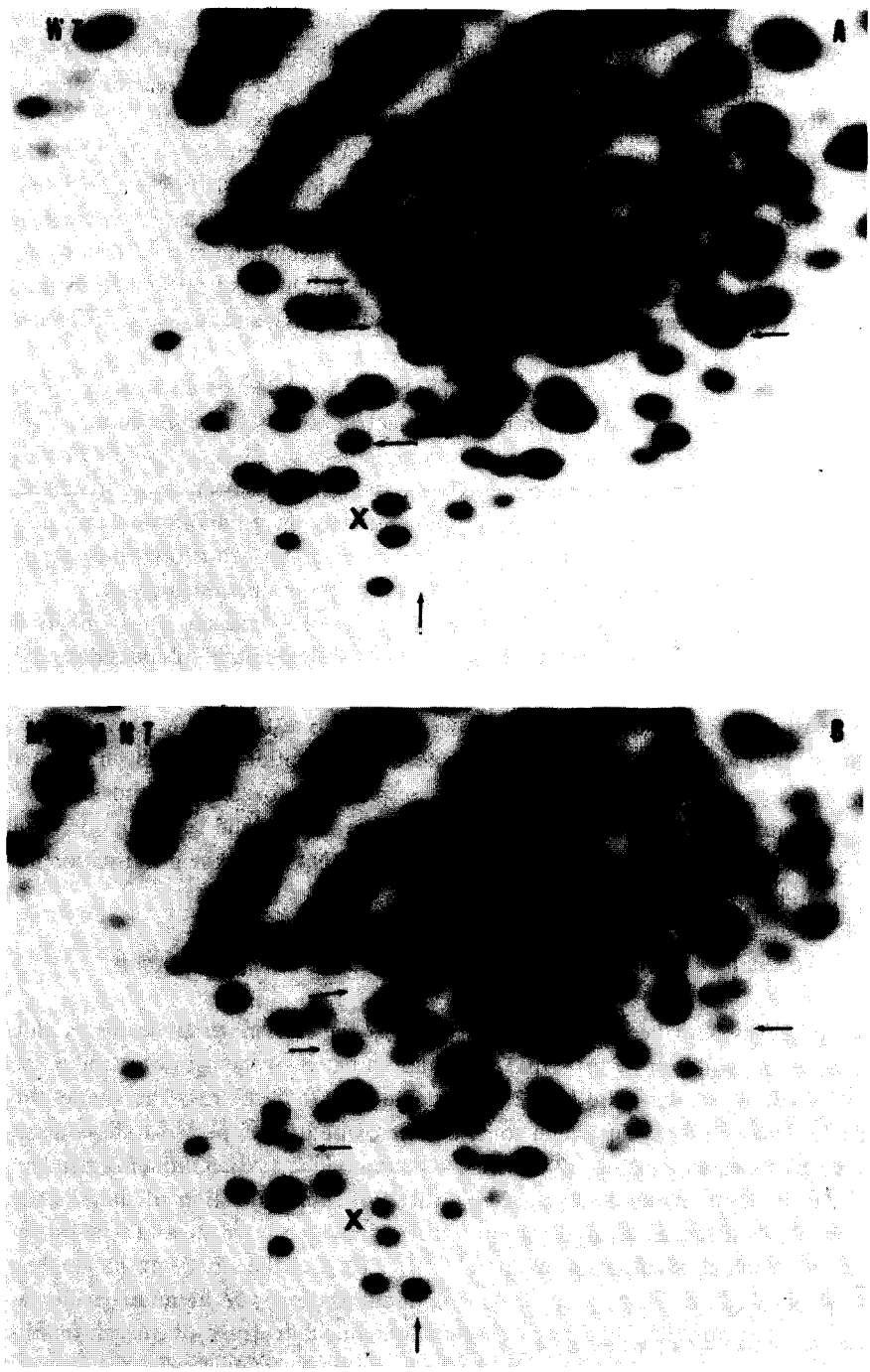


Fig. 1. Oligonucleotide map of virion RNA of *wt* and *ca* A/Ann Arbor/6/60. Differences between the viruses are indicated by arrows.

exhibiting a ⩾4-fold rise in titer of HI antibodies. The A/Leningrad/9/46 (H1N1) *ca* virus was tested in 130 7–14 year old children in 1970, a time when the children would be expected to lack any immunity from prior infection because H1N1 viruses did not circulate between 1957 and 1977. Febrile reactions to the vaccine were reported in 0.7% of the children and a seroconversion rate of approximately 70% was obtained.

In the United States, the *ca* A/Ann Arbor/6/60 (H2N2) virus has never been tested in fully susceptible volunteers. Two studies, however, were done with volunteers known to possess low to moderate levels of neutralizing antibody [13]. In the first study, 6.4×10^4 $TCID_{50}$ of virus was administered to volunteers with serum-neutralizing antibody titers of >16, and no evidence for infection was obtained. In the second study, eight volunteers with serum-neutralizing antibody titers of between 4 and 16 were inoculated with approximately 3×10^5 $TCID_{50}$, and no illnesses were reported; virus was not recovered in the 9 days followed vaccination, but an antibody response was measured in seven of eight subjects 2 weeks after inoculation.

RECOMBINANT *ca* ATTENUATED STRAINS

Method of preparation

Cold-adapted recombinant viruses deriving genes from the Leningrad *ca* strains have been obtained by infection of eggs with approximately 10^6-10^7 EID_{50} of a mixture containing one *ca* virus and one *wt* strain. After mixed infection the eggs are incubated at 32°C for 10 h and subsequently two or three selective passages are performed in the presence of antiserum specific for the *ca* parent. These selective passages are done at either 25°C or 32°C. Final cloning of recombinants is done by triple-plaque purification in chick embryo kidney cell culture or, as a rule, by terminal dilution in chick embryos incubated at a temperature of 32°C. This procedure has reliably selected recombinants having surface antigens of the *wt* viruses and *ca* properties of the Leningrad mutant parent used in the recombination.

Recombinants of the A/Ann Arbor/6/60 virus have been obtained by mixed infection of CK cells at 25°C with approximately 5 p.f.u./cell of each of the two viruses. The infected cells are incubated at 25°C for several days until cytopathic effects are observed and the tissue culture fluid then used as a source of *ca* recombinants. Such recombinants are selected by two sequential passages in CK cells at 25°C in the presence of antiserum to the A/Ann Arbor/6/60 virus followed by two or three plaque purifications in CK cells at 25°C in the absence of sera [33]. To increase the possibility of obtaining recombinants with different gene combinations, it is now customary to establish the mixed infection in 10 or 20 parallel CK cultures which are then maintained independently through all the steps of the selection and cloning procedures [12].

Both in the U.S.S.R. and in the U.S.A. an attempt to increase the reproducibility of the recombination procedure, and to provide a better background for analyzing the effect of recombination on the *wt* virus, has been the cloning of the *wt* virus prior to the

initiation of recombination. This cloning is done by passage of the *wt* virus at 38–40°C in either eggs (U.S.S.R.) or bovine kidney or CK cells (U.S.A.) in order to assure that the parental virus is not *ts*. At least one plaque purification or terminal dilution passage in eggs is undertaken as part of this *wt* virus cloning procedure.

Biological properties of recombinant ca *viruses*

The Leningrad *ca* viruses have been recombined with *wt* influenza A (H1N1) or (H3N2) strains on eight occasions, and *ca* vaccine candidates recovered that have antigens of the contemporary *wt* isolates (Table 6). A majority of the recombinants had a *ts* phenotype similar to that of their Leningrad parent, but in a few instances reduction or loss of *ts* phenotype occurred.

In the U.S.A. *ca* A/Ann Arbor/6/60 has been recombined on nine occasions with *wt* influenza A (H3N2) strains and on four occasions with *wt* influenza A (H1N1) strains [11,12] (also see Table 12). More than 40 of the *ca* recombinants have been analyzed and with a single exception retained a highly *ts* phenotype [12,26,33,36] (and unpublished results). The exception, CR 13 clone 0 which was not *ts* [36], may be improperly cloned as it contains extra RNA segments when analyzed by gel electrophoresis (unpublished observations).

A/Ann Arbor/6/60 recombinant clones intended for use in human volunteer studies are also evaluated in susceptible ferrets infected, as a rule, with 10^7–10^8 EID_{50} of virus (approximately 100–1000 median ferret infectious doses). Histopathological studies are consistent with a milder response by the ferrets to infection with *ca* recombinant than with *wt* virus (Fig. 2). Other markers examined are the ability to replicate well only in the turbinates (but poorly in the lungs), to have low reactogenicity, and not to revert in *ts* or *ca* phenotypes [14,28]. When ferret inoculum is in the range of $10^{7.0}$–$10^{8.0}$ EID_{50} the viruses were, with rare exception, restricted to growth in turbinates, and genetically stable (Table 7). On a single occasion revertant virus was isolated from ferrets infected with CR18 clone 7. Ferrets infected with CR31 clone 3, however, have consistently had virus isolated from lungs, and this virus was not *ts*. The non-*ts* reisolate has been phenotypically stable on subsequent ferret passage, but does not cause disease in the animals, indicating that the *ts* phenotype can alter independently of attenuation in this animal model. The relationship of genetic composition to biological properties of the *ca* recombinants is discussed below.

Infectiousness, reactogenicity and immunogenicity of cold-adapted recombinant viruses

Standardized procedures for safety and immunogenicity (phase 1) tests of *ca* recombinants derived from the A/Ann Arbor/6/60 mutant have been developed that are currently followed by different clinical investigators in the U.S.A. This is done to provide uniform data on clinical reactogenicity, as well as to provide specimens for laboratory testing of virus replication, genetic stability and humoral immune response (Table 8).

TABLE 6

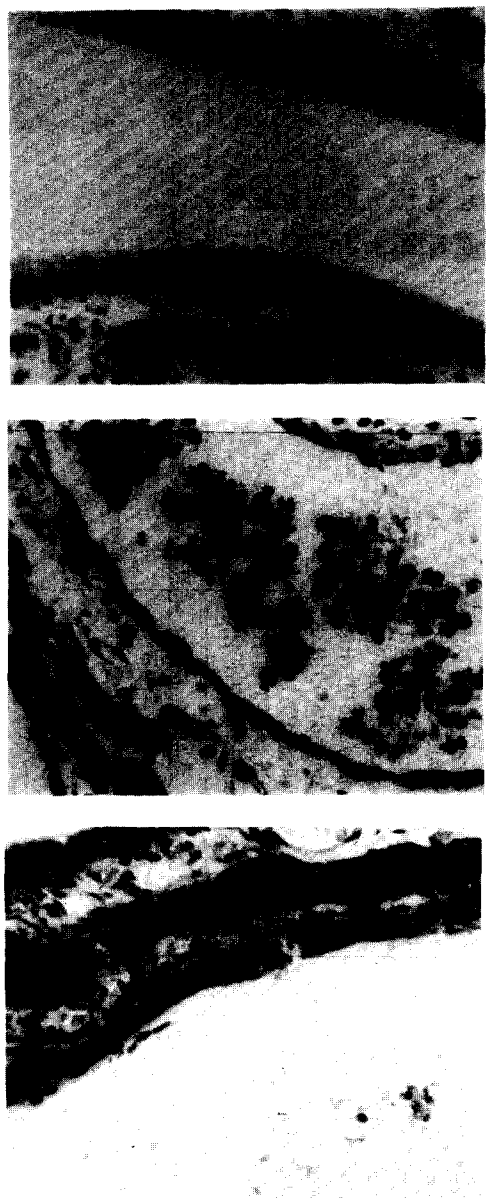
Biological and clinical properties of cold-adapted H3N2 and H1N1 Leningrad recombinant viruses

Vaccine					Response of volunteers[a]		
Cold-adapted parent			Wild-type parent		No. studied	No. (%) with serum HI response	No. with fever >38.5°C
Clone	Strain	25°C passages		*ts* phenotype			
H9	A/Leningrad/134/57(H2N2)	17	A/Victoria/35/72(H3N2)	*ts*	8	8 (100)	0
H24	A/Leningrad/134/57(H2N2)	17	A/Victoria/35/72(H3N2)	*ts*	7	6 (86)	0
H29	A/Leningrad/134/57(H2N2)	17	A/Leningrad/538/74(H3N2)	non-*ts*	6	3 (50)	0
H1	A/Leningrad/134/57(H2N2)	17	A/Victoria/3/75(H3N2)	*ts*	16	4 (25)	1
H5	A/Leningrad/134/57(H2N2)	17	A/Victoria/3/75(H3N2)	*ts*	10	1 (10)	0
H1/4	A/Leningrad/134/57(H2N2)	17	A/Leningrad/82/76(H3N2)	partial[b]	11	9 (82)	0
H17	A/Leningrad/134/57(H2N2)	17	A/Leningrad/82/76(H3N2)	*ts*	30	24 (80)	0
H/47/7/2	A/Leningrad/134/57(H2N2)	47	A/Bangkok/1/79(H3N2)	*ts*	–[c]	– –	–
H25/5	A/Leningrad/134/57(H2N2)	17	A/Khabarovsk/1/77(H1N1)	*ts*	37	28 (77)	0
H25/17	A/Leningrad/134/57(H2N2)	17	A/Khabarovsk/1/77(H1N1)	*ts*	39	21 (54)	1
H32/5	A/Leningrad/134/57(H2N2)	17	A/Khabarovsk/1/77(H1N1)	*ts*	141	98 (70)	0
H47/25/1	A/Leningrad/134/57(H2N2)	47	A/Leningrad/322/79(H1N1)	*ts*	–[c]	– –	–
H3	A/Leningrad/9/46(H1N1)	37	A/Leningrad/5/73(H3N2)	*ts*	20	16 (80)	0
H4	A/Leningrad/9/46(H1N1)	37	A/Leningrad/5/73(H3N2)	*ts*	7	5 (71)	0
H8	A/Leningrad/9/46(H1N1)	37	A/Leningrad/5/73(H3N2)	*ts*	3	2 (67)	0
H4/1	A/Leningrad/9/46(H1N1)	37	A/Leningrad/82/76(H3N2)	partial[b]	50	43 (86)	0
H14	A/Leningrad/9/46(H1N1)	37	A/Leningrad/82/76(H3N2)	*ts*	24	21 (88)	0

[a] Determined for serologically susceptible young adult volunteers administered (by nasal spray) two doses at a 2-week interval, each dose containing approximately $10^{7.0}$ EID_{50}.

[b] Virus yield at 40°C was reduced between 2 and 3 $\log_{10}$ compared to 5 or 6 $\log_{10}$ reduction for other, *ts*, viruses.

[c] Analysis in progress.



Fg. 2. Histopathology of nasal turbinates in ferrets infected with wild-type (*wt*) and cold-adapted (*ca*) recombinant influenza virus. A) Uninfected ferret. B) Ferret infected with *wt* A/Victoria/3/75. At 4 days post-infection there is purulent rhinitis evidenced by the existence of an exudate of inflammatory cells, squamous metaplasia, and oedema of the connective tissue. C) Ferret infected with AA-CR19 clone 0 cold-adapted recombinant of A/Victoria/3/75. At 4 days post-infection localized rhinitis is observed with mild squamous metaplasia, but no purulent exudate.

TABLE 7

Replication in ferrets of cold-adapted influenza viruses derived by recombination with cold-adapted A/Ann Arbor/6/60[a]

Recombinant	Infecting dose	Duration of virus replication (days)		Reversion	Reacto-genicity
		Turbinates	Lungs		
AA-CR 6 clone 0	7.5[b]	3	<2[c]	–	–
AA-CR 18 clone 7	7.5	8	<2	Non-*ca*, non-*ts* virus at 4 days only	–[d]
AA-CR 19 clone 0	7.7	4	<2	–	–[d]
AA-CR 22 clone 1	7.5	4	<2	–	–
AA-CR 29 clone 2	7.7	5	<2	–	–
AA-CR 31 clone 3	8.0	3	3	Stable non-*ts* virus recovered in lungs	–[d]
AA-CR 31 clone 10	7.8	3	<2	–	–
AA-CR 33 clone 1	7.5	3	<2	–	–
AA-CR 35 clone 2	7.5	4	<2	–	–

[a] All wild-type parents of *ca* viruses replicated in ferret lungs and produced fever associated with purulent rhinitis.

[b] Log^{10} EID_{50}.

[c] <1 log^{10} EID_{50} of virus present in 20% lung suspensions prepared from animals sacrificed 48 h post-infection.

[d] Short-lived low-grade fever and histopathological evidence of patchy or diffuse purulent rhinitis observed in some ferrets separately infected with > $10^{8.9}$ EID_{50}.

Expanded safety studies (phase 2) as well as studies of protective efficacy by artificial challenge or natural exposure in field trials (phase 3), are also undertaken. Additional studies have been described by investigators outside the U.S.A. for a few *ca* recombinants. A summary of clinical studies performed with the recombinants is given in Table 9.

Recombinants with H3N2 antigens, administered in phase 1 studies by nose drops at doses of $10^{6.5}$–$10^{7.5}$ $TCID_{50}$ or greater, have infected about 75–100% of vaccinees as judged by virus isolation and serum HI antibody response [14,25,40,42] (Table 10).

In these phase 1 trials usually no more than a single participant (about 10% of infected persons) has experienced more than a mild upper respiratory illness. Febrile or systemic symptoms in those few individuals who experienced them were transient and also not severe. In one trial, however, when undiluted AA-CR 18 virus containing $10^{8.5}$ $TCID_{50}$ of virus was administered, one-third of the volunteers experienced some febrile or systemic reactions [42]. Less pronounced symptoms resulted when a lower vaccine dose was used. Other evidence for possible dose response of reactions was the report that a high dose of AA-CR 22 vaccine was associated with transient afebrile systemic reactions [40], although these have not been seen with a smaller inoculum (R.B. Couch and T. Cates, unpublished results). These results with H3N2 *ca* recombinants of A/Ann

TABLE 8

Protocol for phase 1 (safety) testing of live vaccine in healthy young adults in the U.S.A.

A. General procedure
1. Pre-bleed potential vaccine participants.
2. Test pre-study serum by HI, neutralization, or enzyme-linked immunosorbent (ELISA) assay, to identify volunteers who have maximum susceptibility to the vaccine virus.
3. Quarantine all selected volunteers in restricted access area for 2–3 days to monitor for any developing illness.
4. Under blind conditions, administer by drops vaccine or placebo (0.5 ml/nostril) to supine volunteers. Approximately 3/4 of participants receive vaccine, the remainder placebo.
5. Monitor febrile, systemic and respiratory reactions and collect daily nasal wash specimens.
6. After 2 weeks collect final serum specimen. Volunteers depart from restricted access area.

B. Laboratory tests on specimens collected
1. Inoculation of tube cultures of monkey kidney (MK) or Madin–Darby canine kidney (MDCK) cells at the permissive temperature of 34°C with dilutions of nasal wash specimens to titrate post-inoculation replication of virus, and verify cessation of virus shedding. Additional virus isolation studies are undertaken to diagnose possible non-influenza infections in case of illness in participants.
2. Inoculation of tube cultures of MK or MDCK cells at the non-permissive temperature of 39°C with a low dilution of nasal wash specimens to detect possible revertant (non-*ts*) virus.
3. Titration of virus isolates in primary chicken kidney cells at 25°C, 33°C and 39°C to examine stability of *ca* and *ts* markers.
4. Titration of pre- and post-inoculation sera specimens by HI, and in some cases by neutralization and ELISA procedures, to detect and quantitate humoral antibody responses.
5. In some cases titration of nasal wash specimens by neutralization and ELISA procedures to quantitate local antibody responses.

Arbor/6/60 therefore suggest that, when given at doses of about $10^7 \pm 0.5$ $TCID_{50}$, the viruses are infectious and immunogenic for selected, serologically susceptible, young adult volunteers, but that undiluted vaccines containing 10^8 or greater $TCID_{50}$ may exhibit undesirable, albeit short-lived, reactions.

Open studies, in which participants are enrolled without prescreening of their serologic status, have been done for three H3N2 recombinants of *ca* A/Ann Arbor/6/60. Davenport and collaborators [14,25] administered, by nasal spray rather than drops, $10^{6.5}$ EID_{50} of AA-CR6 to 25 healthy young adult volunteers, and compared doses of $10^{5.5}$, $10^{6.5}$ and $10^{7.5}$ EID_{50} of AA-CR19 administered by nasal spray to groups of approximately 40 young adults. No significant clinical reactions were observed in any study, although the majority of the participants possessed some pre-existing antibody that might have conferred protection against illness induced by the vaccine. In the study with $10^{6.5}$ EID_{50} of AA-CR6 vaccine, 15/19 (79%) of subjects with pre-existing HI antibody titers of <64 exhibited a 4-fold antibody response. The study with AA-CR19 demonstrated 4-fold or greater antibody responses in 10/28 (36%), 15/26 (58%) and 13/18 (72%) of volunteers with pre-existing HI titer <100 following inoculation with $10^{5.5}$,

TABLE 9

Human volunteer studies undertaken during development and evaluation of cold-adapted viruses prepared by recombination at the University of Michigan with A/Ann Arbor/6/60 mutant

Recombinant	Wild-type parent	Safety and immunogenicity				Protective efficacy	
		Phase 1[a]		Phase 2[b]		Closed[c]	Open[d]
		Adults	Children	Adults	Children		
	H3N2						
AA-CR 6 clone 0	A/Queensland/6/72	14[e]					
AA-CR 18 clone 7	A/Scotland/840/74	42					
AA-CR 19 clone 0	A/Victoria/3/75	42, *		25		*	
AA-CR 22 clone 1	A/Victoria/3/75	40, *				*	
AA-CR 29 clone 2	A/Alaska/6/77	41, *	***				*
AA-CR 31 clone 3	A/Alaska/6/77	41, *					
AA-CR 31 clone 10	A/Alaska/6/77	41					
	H1N1						
AA-CR 33 clone 1	A/USSR/90/77	51					
AA-CR 35 clone 2	A/Hong Kong/123/77	44	29	*		*	*
AA-CR 37 clone 1	A/California/10/78	**	†				

[a] Small studies, with usually isolated volunteers, involving up to 25 persons vaccinated with any dose of virus. Individuals are examined daily for 1 or 2 weeks for clinical symptoms and determinants of virus replication.

[b] Larger studies, involving about 100–200 persons, not necessarily isolated, but with daily examination for 1 week of each participant for clinical symptoms. Accumulated results of phase 1 studies were substituted for a phase 2 study with AA-CR 29 clone 2.

[c] Small groups of isolated volunteers, previously vaccinated, are challenged with a standard dose of wild-type virus and monitored for clinical symptoms and evidence of virus replication.

[d] Several hundred volunteers, vaccinated prior to the influenza season, provide illness records during the influenza epidemic and provide pre- and post-epidemic serum specimens to determine infection rate. Participants experiencing influenza-like illness during the epidemic are encouraged to provide a culture for virus isolation at the time of their acute illness.

[e] Reference numbers: *R.B. Couch and T. Cates, unpubl. results; ** G. Douglas and R.E. Betts, unpubl. results; ***P. Wright, unpubl. results; †B. Belshe, unpubl. results.

TABLE 10

Infectiousness, immunogenicity and reactogenicity of cold-adapted H3N2 A/Ann Arbor recombinant vaccines in phase 1 studies with young adult volunteers

H3N2 recombinants	Dose (log_{10} $TCID_{50}$)	No. of volunteers	No. (%) infected[a]	No. (%) infected with		Reference
				HI response	Febrile or systemic illness	
CR 18 clone 7	7.5	10	8 (80)	6 (75)	1 (13)	50
	8.5	12	12 (100)	11 (92)	4 (33)	50
CR 19 clone 0	6.3	14	13 (93)	13 (93)	0 (0)	64
	7.5	13	12 (92)	10 (83)	1 (8)	50
CR 22 clone 1	6.8	9	9 (100)	9 (89)	1 (11)	64
CR 29 clone 2	7.5	24	18 (75)	16 (89)	0 (0)	61
CR 31 clone 3	7.7	12	12 (100)	7 (58)	0 (0)	61
CR 31 clone 10	7.7	17	13 (76)	10 (77)	1 (8)	61
H1N1 recombinant						
CR 35 clone 2	7.5	25	24 (96)	11 (46)[b]	0 (0)	53

[a] Determined by virus isolation and antibody response.

[b] Increased to 100% by use of immunoassay to detect serum IgG and IgM antibody responses.

$10^{6.5}$ and $10^{7.5}$ EID_{50} of virus, respectively. These results suggest that doses of about $10^{6.5}$–$10^{7.5}$ EID_{50} of virus infect the majority of susceptible volunteers when given by spray. Studies are now planned to determine the median human infectious dose of cold-adapted A/Ann Arbor/6/60 recombinant vaccines in volunteers with low pre-existing antibody, when vaccine is administered by nasal drops (B. Murphy, personal communication).

One open-field trial has been undertaken with AA-CR 29 clone 2 recombinant, in which groups of more than 200 college students received vaccine or placebo under blind conditions. No differences in respiratory illness rates reported by the students (21–29%) were seen between the groups within the week following vaccination (R. Couch and T. Cates, personal communication). Serologic responses and protection against natural infection are still under evaluation.

Cold-adapted H1N1 recombinants have also been evaluated in human volunteer tests. Phase 1 clinical studies using similar protocols to those developed with H3N2 candidate vaccines were undertaken in the U.S.A. with a *ca* recombinant, AA-CR-35, which derives its HA and NA antigens from an A/USSR/90/77(H1N1)-like *wt* virus strain [44]. All of 24 selected serologically susceptible volunteers were infected when a virus dose of $10^{7.5}$ $TCID_{50}$ was administered (Table 10). Maximum detection of infection required the use of an enzyme immunoassay [46] to measure serum antibody responses, because only 45% of the participants responded to vaccination with a detectable serum HI antibody response. Similarly to the H3N2 *ca* recombinants, AA-CR 35

did not cause significant clinical reactions when given at doses of $10^{7.5}$ $TCID_{50}$ or less. Comparable results were obtained with an additional recombinant (AA-CR33) of A/USSR/90/77(H1N1)-like virus evaluated in Europe in young volunteers [51], and with a recombinant independently prepared in Europe from the A/Ann Arbor/6/60 *ca* parent [51,61].

Clinical studies in the U.S.A. of the AA-CR35 H1N1 *ca* recombinant in healthy young adults include a phase 2 study involving about 100 persons, and an open field trial in which about 700 college students were vaccinated. In these larger studies, no significant reactions were observed compared to the placebo groups, but the vaccine immunogenicity, as judged by conventional HI tests, was only about 50–60% in serologically susceptible vaccinees (R. Couch and T. Cates, personal communication). This does not necessarily indicate low infectivity of the *ca* recombinant, because natural infection similarly was associated with relatively poor antibody response in young adults during 1977 and 1978, when measured with conventional HI tests [22].

Because of observations of low reactogenicity in young adults, AA-CR35 vaccine was considered safe for testing in small numbers of young children of 13–44 months old [29]. When a dose of $10^{6.5}$ $TCID_{50}$ was administered to 11 children, eight (73%) were infected, all of these shedding virus (for up to 13 days) and demonstrating serum HI antibody response. No febrile or systemic reactions were observed (Dr. P. Wright, personal communication), and all virus isolates retained their *ts* phenotype.

Attempts to demonstrate protective efficacy of the *ca* recombinants have been undertaken by R. Couch and T. Cates (Personal communication), including the above-mentioned two open field trials, and studies where a small number of vaccinated volunteers are challenged with *wt* virus under conditions similar to those used for the initial vaccination. To obtain maximum sensitivity in determining vaccine efficacy in the prevention of influenza illness, laboratory diagnosis of viral infection is undertaken to verify that any illnesses are caused by influenza virus, and placebo groups are included. A total of 25 volunteers vaccinated 1–3 months previously with *ca* H3N2 or H1N1 recombinants have been challenged with *wt* virus without any evidence of illness, whereas 37 controls experienced 50–68% illness rates (Table 11). Results of open field trials are still being evaluated.

Preliminary clinical evaluations in the U.S.S.R. of the *ca* viruses derived by recombination with the Leningrad *ca* parental strains are done by immunizing healthy children or young adults, 17–25 years old, residents in communes. Pre-vaccine sera are collected and tested to identify vaccinees who have serum HI antibody titers of $\leqslant 8$ to the vaccine strains. Some volunteers are also shown to lack detectable antineuraminidase antibody. The protocols are similar to those described above for evaluation of donor Leningrad *ca* strains.

Results indicate that in most instances greater than two-thirds of the serologically susceptible participants (i.e. whose pre-vaccine serum HI titer is $\leqslant 8$) were infected as judged by serum HI antibody response measurement (Table 6). Recombinants obtained with A/Victoria/3/75 *wt* virus, however, were poorly infectious and immunogenic, and

TABLE 11

Human volunteer studies[a] to demonstrate protective efficacy of cold-adapted recombinant influenza vaccines in closed trial with virulent *wt* virus challenge[b]

Subjects			Challenge virus	Response to challenge	
Previous vaccination or infection with	No.	Range of pre-challenge serum neutralization antibody titers		% infected	% ill[c]
None known (control)	25	<2	A/Victoria/3/75(H3N2)	96	68
AA-CR 19 clone 0	12	<2–32	A/Victoria/3/75(H3N2)	33	0
AA-CR 22 clone 1	6	<2–256	A/Victoria/3/75(H3N2)	17	0
None known (control)	12	<2–12	A/Hong Kong/123/77(H1N1)[d]	75	50
AA-CR 35 clone 2	7	<2–128	A/Hong Kong/123/77(H1N1)	29	0

[a] Results of Drs. R.B. Couch and T. Cates.

[b] Challenge dose was $10^{5.25}$ $TCID_{50}$ for A/Victoria/3/75 virus and $10^{3.75}$ $TCID_{50}$ for A/USSR/90/77-like virus.

[c] Mainly febrile or systemic illness and upper respiratory symptoms.

[d] Antigenically similar to A/USSR/90/77.

this may be attributable to the fact that the *wt* A/Victoria/3/75 parent used in the recombination experiments in the Soviet Union had undergone a spontaneous mutation during laboratory passage, and in contrast to the A/Victoria/3/75 *wt* used in the U.S.A. had only moderate reactogenicity and immunogenicity [50]. The poor immunogenicity suggested by the results for the recombinant clone H29 derived from A/Leningrad/538/74 might be solely due to the small numbers tested.

For the H1N1 recombinant clones neuraminidase antibody responses following vaccination were also measured, to provide an additional indicator of immunogenicity. In initially seronegative participants, neuraminidase antibody response was detected for 37%, 24% and 15%, respectively, with clones H32/5, H25/17 and H25/5. All of the tested vaccines were highly attenuated, and clone H32/5 which had the highest overall immunogenicity was therefore selected for use in vaccine manufacture.

Clones H47/7/2 and H47/25/1 which were attenuated by recombination between A/Bangkok/1/79(H3N2) or A/Leningrad/322/79(H1N1) *wt* strains respectively and the 47-times, 25°C-passaged A/Leningrad/134/57(H2N2) *ca* mutant are presently being evaluated in 7–14 year old children, with preliminary results indicating a reactogenicity and high immunogenicity (M.R. Zykov, personal communication).

Studies of protective efficacy by the *ca* Leningrad recombinants are not done by means of artificial challenge of volunteers immunized with vaccine or placebo, but by monitoring reported illness rates among vaccinated and non-vaccinated groups during the winter season. Under these circumstances, when laboratory diagnosis of infection is not done, vaccine efficacy in reducing acute respiratory disease is dependent on the proportion of influenza to non-influenza infections in the communities studied (Uy.G. Ivannikov, personal communication).

Effect of genome composition on properties of ca *recombinant live-vaccine candidates*

Genome analyses of *ca* viruses derived by recombination with *ca* A/Ann Arbor/6/60 at 25°C have revealed them to have a highly restricted composition. Of 40 clones examined, 24 (61%) possessed only the HA and NA of *wt* virus, but all other genes from the *ca* A/Ann Arbor/6/60 parent (Table 12) [11,12]. The remaining viruses always contained P2, P3 and nucleoprotein genes from the Ann Arbor *ca* parent, but a single one of the P1, matrix protein or non-structural protein genes from the *wt* parent. Several recombinants with *wt* P1 or non-structural proteins were recovered, but have not been found to differ markedly in any biological property from recombinants with all non-HA- and non-NA genes derived from the *ca* A/Ann Arbor/6/60 parent (Table 7) [12,26,27]. However, relative temperature sensitivity of A/Ann Arbor/6/60 recombinants can apparently vary somewhat, independently of gene combinations [43].

Only a single recombinant derived at 25°C was determined to have a *wt* matrix protein. As described above (Table 7), this recombinant (AA-CR 31 clone 3) exhibited instability of its *ts* phenotype in ferrets, supporting the view from results with recombinants derived at temperatures other than 25°C that matrix protein was involved in the

TABLE 12

Gene composition of cold-adapted Ann Arbor viruses derived by recombination at 25°C

Recombinants			HA	NA	Derivation of genes[a]					
Expt.	Wild-type parent	Clone			P3 (RNA 1)[b]	P1 (RNA 2)[b]	P2 (RNA 3)[b]	NP	M	NS
AA-CR 6	A/Queensland/6/72(H3N2)	0	W	W	A	A	A	A	A	A
AA-CR 12	A/Ann Arbor/9/73(H3N2)	0	W	W	A	A	A	A	A	A
AA-CR 13	A/Dunedin/4/73(H3N2)	5	W	W	A	W	A	A	A	A
		9	W	W	A	W	A	A	A	A
AA-CR 18	A/Scotland/840/74(H3N2)	0	W	W	A	A	A	A	A	W
		4	W	A	A	A	A	A	A	W
		5	W	A	A	A	A	A	A	W
		6	W	W	A	A	A	A	A	W
		7	W	W	A	A	A	A	A	W
AA-CR 19	A/Victoria/3/75(H3N2)	0	W	W	A	W	A	A	A	A
AA-CR 22	A/Victoria/3/75(H3N2)	1	W	W	A	W	A	A	A	A
		17	W	A	A	A	A	A	A	A
AA-CR 29	A/Alaska/6/77(H3N2)[c]	2	W	W	A	A	A	A	A	A
		17	W	W	A	A	A	A	A	A
AA-CR 31	A/Alaska/6/77(H3N2)[c]	2	W	W	A	A	A	A	A	A
		3	W	W	A	A	A	A	W	A
		4	W	A	A	A	A	A	A	A
		10	W	W	A	A	A	A	A	W
		12	W	W	A	A	A	A	A	W
		13	W	W	A	A	A	A	A	A
		16	W	A	A	A	A	A	A	A
		17	W	A	A	A	A	A	A	A
		19	W	W	A	A	A	A	A	A
		20	W	W	A	A	A	A	A	A
AA-CR 33	(A/USSR/90/77(H1N1)	1	W	W	A	A	A	A	A	A
		2	W	W	A	A	A	A	A	A
AA-CR 35	A/Hong Kong/123/77(H1N1)[c]	2	W	W	A	A	A	A	A	A
AA-CR 36	A/Hong Kong/123/77(H1N1)[c]	1	W	W	A	A	A	A	A	A
		2	W	W	A	A	A	A	A	A
		3	W	W	A	A	A	A	A	A
		4	W	A	A	A	A	A	A	A
		5	W	W	A	A	A	A	A	A
		7	W	W	A	A	A	A	A	A
		9	W	W	A	A	A	A	A	A
		10	W	W	A	A	A	A	A	A
AA-CR 37	A/California/10/78(H1N1)	1	W	W	A	A	A	A	A	A
AA-CR 44	A/Beijing/2/79(H3N2)[c]	6	W	W	A	A	A	A	A	A
		11	W	W	A	A	A	A	A	W
		16	W	W	A	A	A	A	A	A
		20	A	W	A	A	A	A	A	A

[a] W indicates gene derived from wild-type parent. A indicates gene derived from mutant parent.

[b] For reference conditions of electrophoresis without urea at 38°C [26].

[c] Adapted to growth in primary bovine kidney cells and cloned at 39°C in these cells.

expression of the A/Ann Arbor/6/60 *ts* phenotype. AA-CR 31 clone 3 did not exhibit unusual properties in a human volunteer study, however, and non-*ts* revertants were not detected amongst isolates recovered from the vaccinees [41].

Occasionally viruses exhibiting partial reversion in either the *ts* or the *ca* phenotype (but not both phenotypic markers) have been identified as a small proportion of virus isolated from human volunteers or hamsters infected with Ann Arbor *ca* recombinants [42,55]. There has not yet been an examination of genetic changes or changes in human virulence, that might be exhibited by such partial revertants.

The two *ca* recombinants used in the U.S.S.R. as live vaccines in public immunization programs in recent years, i.e. the H17 clone of A/Leningrad/82/76(H3N2), and the 32/5 clone of A/Khabarovsk/1/77(H1N1), both contain only the HA and NA of their respective *wt* parent, but all six other genes derived from the A/Leningrad/134/57 (H2N2) *ca* parent [17] (Table 13). Recombinant clones H47/7/2 and H47/25/1 which were recently prepared using the 47-times, 25°C-passaged A/Leningrad/134/57(H2N2) parent similarly derived only their HA and NA genes from their *wt* parents. Two other recombinants, H/1/4 and H/4/1 contained at least all seven genes other than the M protein from that *wt* parent and were non-*ts* or partially *ts* respectively. The matrix protein gene in recombinant H/1/4 could not be identified by the procedure used as it appeared to have undergone a spontaneous mutation during recombination and was not identical to the matrix protein in either parent. Results of genome analysis suggest that the matrix protein is probably not required for the *ca* phenotype of either A/Leningrad/134/57 or A/Leningrad/9/46 *ca* donors, but is involved in the *ts* phenotype of these viruses. Comparison of genotype with results of clinical testing [17] (Tables 6 and 13) suggests that even those recombinants possessing only a single gene (M) from the *ca* parent were attenuated. Evaluation of genetic stability of recombinants during replication in human volunteer studies has been done for the H17 clone which contains all non-HA, non-NA genes from its *ca* parent. Eight isolates were obtained from vaccinees shedding virus and all retained the *ts* phenotype, although the difference in titers at 40°C and 32°C for the isolates was 1–2 $\log_{10}$ less than in the original vaccine.

SUMMARY AND CONCLUSIONS

In the Soviet Union and the United States *ca* variants of influenza A virus have been selected by multiple passage at temperatures suboptimal for replication of *wt* virus. These *ca* variants replicate efficiently at 25°C or 28°C but are highly temperature-sensitive and unlike many *wt* influenza A viruses will not replicate at 39–40°C.

The *ca* mutant most widely used for preparing experimental vaccine strains in the U.S.A. is Ann Arbor/6/60 (H2N2), but due to a lack of serologically susceptible adult volunteers this variant has itself been only partially characterized for clinical and immunological response in man. However, a series of recombinants deriving five or six of the non-HA, non-NA genes from the A/Ann Arbor/6/60 mutant, but HA, NA and sometimes one other, genes from *wt* H3N2 or H1N1 strains have been tested in serologically susceptible human volunteers. When given by nose drop or spray at doses of about 10^7 $TCID_{50}$ or EID_{50} the viruses are highly attenuated. H3N2 recombinants are highly immunogenic, but H1N1 recombinants which are also attenuated have often appeared less immunogenic

TABLE 13

Genome composition of recombinant cold-adapted viruses obtained from Leningrad cold-adapted donors and wild-type strains

Recombinant clone	Cold-adapted parent		Wild-type parent		Genome composition[a]							
	Strain	25°C passages		*ts* phenotype	P3 (RNA 1)	P1 (RNA 2)	P2 (RNA 3)	HA	NP	NA	M	NS
H/17	A/Leningrad/134/57(H2N2)	17	A/Leningrad/82/76(H3N2)	yes	L	L	L	W	L	W	L	L
H/1/4	A/Leningrad/134/57(H2N2)	17	A/Leningrad/82/76(H3N2)	partial[b]	W	W	W	W	W	W	ND	W
H/47/7/2	A/Leningrad/134/57(H2N2)	47	A/Bangkok/1/79(H3N2)	yes	L	L	L	W	L	W	L	L
H/25/5	A/Leningrad/134/57(H2N2)	17	A/Khabarovsk/1/77(H1N1)	yes	L	L	L	W	W	W	L	W
H/25/17	A/Leningrad/134/57(H2N2)	17	A/Khabarovsk/1/77(H1N1)	yes	W	L	W	W	W	W	L	L
H/32/5	A/Leningrad/134/57(H2N2)	17	A/Khabarovsk/1/77(H1N1)	yes	L	L	L	W	L	W	L	L
H/47/25/1	A/Leningrad/134/57(H2N2)	47	A/Leningrad/322/79(H1N1)	yes	L	L	L	W	L	W	L	L
H/4/1	A/Leningrad/9/46(H1N1)	37	A/Leningrad/82/76(H3N2)	partial[b]	W	W	W	W	W	W	L	W
H/14	A/Leningrad/9/46(H1N1)	37	A/Leningrad/82/76(H3N2)	yes	L	W	L	W	W	W	L	W

[a] Determined by gel electrophoresis of duplex RNAs obtained by hybridizing virion RNA of wild-type viruses with complementary RNA of recombinants, and comparing migration rates with homologous duplex RNA hybrids obtained when both RNA strands derive from the same parental virus. L. gene inherited from Leningrad cold-adapted parent; W, gene inherited from wild-type parent; ND, not determined because electrophoretic mobility of duplex RNA did not correspond with homologous duplex RNA of either parent [17].

[b] 40°C virus yield was reduced between 2 and 3 $\log_{10}$, compared to 5 or 6 $\log_{10}$ reduction for other *ts* viruses.

when traditional assays for antibody response are used. Preliminary studies of protective efficacy using artificial challenges have been undertaken with encouraging results in small numbers of volunteers.

Genetic and biochemical analysis has identified the existence of mutations in all eight genes of the *ca* A/Ann Arbor/6/60 virus, and those coding for the polymerase-associated proteins P1, P2 and P3, as well as the matrix protein have been implicated in some way with genetic or phenotypic in vitro markers, although the relevance to attenuation of any mutation detected by these means is unclear. Occasional partial revertants in *ca* or *ts* phenotype have been shed by persons or animals infected with Ann Arbor-*ca* recombinants. Further studies are necessary to determine whether reversion of *ca* or *ts* phenotypes is associated with increased virulence. Production of reproducible *ca* recombinants has probably been facilitated by their ability to produce plaques at 25°C which provides a positive selection mechanism. Techniques used consistently identify with high frequency recombinants possessing all non-HA, non-NA genes from their *ca* A/Ann Arbor/6/60 parent.

In the U.S.S.R. many findings are analogous to those in the U.S.A. The *ca* mutant most widely used for preparing vaccine strains, A/Leningrad/134/57, is also an H2N2 virus similar to A/Ann Arbor/6/60, although another *ca* parent, A/Leningrad/9/46(H1N1), is sometimes used. Both viruses have been tested for their infectiousness, immunogenicity and reactogenicity by administration of 10^7 infectious units with a nasal spray to young children, and were reported to be attenuated and immunogenic. A series of recombinants prepared with these *ca* parents and diverse *wt* parents usually shared the important biological properties of the *ca* parent, and the recombinants with properties most suitable for vaccine use possess all non-HA, non-NA genes of their *ca* parent.

Genetic analysis indicates the existence of mutations in several genes of the Leningrad *ca* parents and implicates the matrix protein as containing a mutation necessary for temperature sensitivity. Revertant viruses have not yet been identified as being shed by vaccinated persons in the small number of instances this has been evaluated. Studies of protective efficacy by artificial challenge of vaccinated volunteers with virulent *wt* virus are not possible in the Soviet Union, and analysis of vaccine efficacy is dependent on epidemiological observations of large population groups during periods of natural virus circulation.

The common viewpoint is held by the authors that the identification of multiple mutations in *ca* parent strains following their selection in the laboratory, and the ability to transfer most or all of these genes to contemporary *wt* strains by recombination (reassortment), facilitates the regular production of *ca* recombinants with reproducible biological properties and minimal chances of reverting to virulence, even though the actual genetic basis for attenuation is unknown, in common with other live virus vaccines. A possible effect of HA and NA genes from the *wt* parent or the recombinants is not totally excluded, however. Studies of the relative efficacy of live attenuated vaccines, compared to inactivated vaccines, in populations with different immunological backgrounds and when exposed to different *wt* virus strains have yet to be done. Continued

development, evaluation, and comparison of live and inactivated influenza vaccines must continue as one approach to improving influenza prophylaxis, and at the present time *ca* influenza viruses are highly useful in preparing live vaccines for this purpose.

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