INFECTION AND IMMUNITY, Aug. 1980, p. 348-355
0019-9567/80/08-0348/08$02.00/0
Vol. 29, No. 2

Evaluation of Influenza A/Hong Kong/123/77 (H1N1) *ts*-1A2 and Cold-Adapted Recombinant Viruses in Seronegative Adult Volunteers

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Two attenuated influenza A donor viruses, the A/Udorn/72 *ts*-1A2 and the A/Ann Arbor/6/60 cold-adapted (*ca*) viruses, are being evaluated for their ability to reproducibly attenuate each new variant of influenza A virus to a specific and desired level by the transfer of one or more attenuating genes. Each of these donor viruses has been able to attenuate influenza A viruses belonging to the H3N2 subtype by the transfer of one or more attenuating genes. To determine whether these two donor viruses could attenuate a wild-type virus that belonged to a different influenza A subtype, *ts*-1A2 and *ca* recombinants of a wild-type virus representative of the A/USSR/77 (H1N1) Russian influenza strain were prepared and evaluated in adult doubly seronegative volunteers at several doses. The recombinants derived from both donor viruses were attenuated for the doubly seronegative adults. Less than 5% of infected vaccinees developed a febrile or systemic reaction, whereas five of six recipients of wild-type virus developed such a response. The 50% human infectious dose (HID_{50}) for each recombinant was approximately $10^{5.0}$ 50% tissue culture infective doses. The virus shed by the *ts*-1A2 and *ca* vaccinees retained the *ts* or *ca* phenotype, or both. This occurred despite replication of the recombinant viruses for up to 9 days. No evidence for transmission of the *ca* or *ts*-1A2 recombinant virus to controls was observed. A serum hemagglutination inhibition response was detected in less than 50% of the infected vaccinees. However, with the more sensitive enzyme-linked immunosorbent assay, a serological response was detected in 100% of the *ca* vaccinees given 300 HID_{50} and approximately 70% of *ca* or *ts* vaccinees who received 10 to 32 HID_{50} of virus. These results indicate that the recombinants derived from both donor viruses were satisfactorily attenuated and were stable genetically after replication in doubly seronegative adults although they induced a lower serum hemagglutination inhibition response than that found previously for H3N2 *ts* and *ca* recombinants.

Attenuation of new strains of influenza A virus can be accomplished rapidly by transferring attenuating genes from an attenuated donor virus to each new epidemic or pandemic strain by genetic reassortment (14). In the United States, temperature-sensitive (*ts*) and cold-adapted (*ca*) donor viruses are being evaluated for their ability to reproducibly attenuate each new variant of influenza A virus to a specific and desired level by the transfer of one or more attenuating genes (14). One donor virus, the influenza A/Udorn/72-*ts*-1A2 virus, is a temperature-sensitive recombinant virus that has a 37°C shutoff temperature of plaque formation in vitro and has *ts* mutations on the genes coding for the P1 and P3 polymerase proteins (10, 18). The *ts* mutations in this virus were originally induced by mutagenesis of its parent viruses with flurouracil (18, 24). The A/Udorn/72-*ts*-1A2 parent virus and its A/Victoria/75-*ts*-1A2 (H3N2), A/Alaska/77-*ts*-1A2 (H3N2), and A/Hong Kong/77 (A/HK/77)-*ts*-1A2 (H1N1) recombinants that possessed both *ts*-1A2 *ts* lesions exhibited a 10,000-fold reduction of virus replication in the lungs of hamsters and a 100-fold reduction in the nasal turbinates (19, 20; B. R. Murphy, N. T. Hosier, and R. M. Chanock, unpublished observations). These results indi-

cated that the two *ts*-1A2 *ts* genes could impose a specific and reproducible restriction of viral replication in animals. The A/Victoria/75-*ts*-1A2 (H3N2) and A/Alaska/77-*ts*-1A2 (H3N2) recombinants have subsequently been found to be satisfactorily attenuated in adult seronegative volunteers (11, 12).

The second donor virus, the A/Ann Arbor/6/60 (A/AA/6/60) *ca* virus, also has a 37°C shutoff temperature for plaque formation and is cold adapted; i.e., it can replicate and produce plaques with much greater efficiency than wild-type virus in chick kidney monolayer cultures incubated at 25°C (7, 8). This donor virus was produced by multiple passages at successively lower temperatures (33 to 25°C) in primary chick kidney cultures (8). The A/AA/6/60 *ca* virus gene(s) that confers the *ts* and the *ca* phenotype and those that confer attenuation have not been identified. However, it is believed that they reside on one or a combination of the RNA1, RNA3, and *NP* genes because these genes are present in all *ca* recombinants produced to date that possess both the *ca* and *ts* phenotypes (1). The transfer of five or six genes from this donor virus to several wild-type viruses was regularly achieved during gene reassortment at 25°C and resulted in attenuation of such recombinants for ferrets (1, 2, 9). Evaluation of three H3N2 *ca* recombinants, A/Queensland/72, A/Victoria/75, and A/Alaska/77, in seronegative volunteers indicated that these were satisfactorily attenuated and antigenic (2, 4, 13). In contrast, an A/Scotland/74 *ca* recombinant virus retained the capacity to induce febrile disease at doses of $10^{7.5}$ 50% tissue culture infective doses ($TCID_{50}$) and higher, although this febrile illness was milder and of shorter duration than that caused by wild-type virus (13).

To determine whether these two donor viruses could attenuate a wild-type virus that belonged to a different influenza A subtype, *ts*-1A2 and *ca* recombinants of a wild-type virus (A/HK/123/77) that was antigenically representative of the A/USSR/77 (H1N1) "Russian" influenza strain were prepared and evaluated in adult seronegative volunteers at several doses. The adult volunteers were selected by age and by serological status so that they lacked known experience with both the H1 hemagglutinin (HA) and N1 neuraminidase (NA); i.e., they were doubly seronegative. The majority of these volunteers were born after the disappearance of H1N1 strains during 1957 and had not been infected with H1N1 virus during the 1977-78 influenza season. The recombinant vaccine viruses, therefore, should replicate in these hosts without restriction resulting from immunity to the HA and NA antigens and thus provide a stringent test of the level of residual virulence and of the antigenicity of the attenuated viruses. The results indicate that the recombinants derived from both donor viruses were satisfactorily attenuated for doubly seronegative adults although their antigenicity was less than that found previously for H3N2 recombinants (11-13).

MATERIALS AND METHODS

Viruses. The A/HK/123/77 (H1N1) wild-type virus, antigenically similar to the A/USSR/90/77 prototype strain, was isolated from throat swab material in the allantoic cavity of specific-pathogen-free embryonated hen eggs. The virus was then passaged twice in primary calf kidney (BK) cultures at 34°C and cloned by plaque passage in monolayer cultures at 34°C and then at 39°C as described (11). The virus was passaged once more at 37°C in BK cultures and inoculated into the allantoic cavity of the embryonated hen egg (Spafas, Inc., Norwich, Conn.). This A/HK/123/77 virus suspension (passage history of egg 1, BK-5, egg 1) titered $10^{7.7}$ $TCID_{50}$/ml in Madin-Darby canine kidney (MDCK) monolayer cultures. The cloned wild-type virus was used as the wild-type parent in matings with the donor viruses and was evaluated for virulence in volunteers in the present study.

The preparation of the A/HK/123/77-*ts*-1A2 clone 144 virus (passage history after recombination BK-3, egg 1) has been described (B. R. Murphy et al., submitted for publication). This virus possessed a 37°C shutoff temperature and both *ts*-1A2 *ts* lesions (Murphy et al., submitted for publication). The virus was cloned at 34°C in BK cultures and inoculated into the allantoic cavity of embryonated hen eggs. This suspension had a titer of $10^{6.8}$ $TCID_{50}$/ml in MDCK cultures.

The A/HK/123/77 *ca* recombinant virus (CR-35, clone 2) was produced by mating the A/AA/6/60 (H2N2) *ca* virus with the cloned A/HK/123/77 (H1N1) wild-type virus, and the *ca* recombinant was selected and cloned as described previously (1, 9). The CR-35 clone 2 virus had a 37 to 38°C shutoff temperature of plaque formation in MDCK monolayer cultures and received all genes except those coding for the HA and NA proteins from the A/AA/6/60 *ca* parent (1). The virus suspension (passage history after recombination, chick kidney, egg 1) titered $10^{7.8}$ $TCID_{50}$/ml on MDCK monolayer cultures.

Studies in volunteers. Volunteers were 25 years old or younger (most were less than 20 years old) and had a hemagglutination inhibition (HAI) antibody titer of ≤1:8 and a neuraminidase inhibition (NI) antibody titer of ≤1:2. The facilities for isolation of the volunteers, the collection and testing of specimens, and the methods for evaluation of clinical response have been described (11, 15). For the HAI test, the split vaccine A/USSR/92/77 (Parke, Davis & Co., Detroit, Mich.) was used as antigen to increase the sensitivity of detection of antibody (5; G. Noble and A. Kendal, unpublished observations). For the NI test, a recombinant was used that had the hemagglutinin of the A/equine 1/Prague/56 virus and the neuraminidase of the A/USSR/92/77 virus. This recombinant was incubated with the fetuin substrate at 30°C rather

than at 37°C because preliminary analysis suggested that the N1 enzyme was more stable at 30°C than at 37°C during the overnight incubation period. To determine whether a serological response occurred in vaccinees, the highly sensitive enzyme-linked immunosorbent assay (ELISA) was used, with whole inactivated A/USSR/92/77 as antigen (16). The sera from volunteers who did not develop an HAI antibody response were tested by ELISA with a goat anti-human immunoglobulin G (IgG) enzyme conjugate. Sera that were negative with the anti-IgG conjugate were then tested with an anti-IgM conjugate as described previously (16).

RESULTS

Evaluation of A/HK/123/77 (H1N1) wild-type virus. Six adult volunteers received $10^{4.2}$ $TCID_{50}$ of cloned wild-type virus, and each was infected as determined by recovery of virus (Table 1). Five of the volunteers developed a febrile response or systemic symptoms, or both, which lasted for 48 to 96 h. This indicated that the A/HK/77 wild-type virus was virulent, and therefore attenuation resulting from the acquisition of genes derived from the *ts* and *ca* donor viruses could be determined.

Attenuation of the A/HK/123/77-*ts*-1A2 and -CR-35 recombinants for adults. The *ts*-1A2 and CR-35 viruses were administered at several doses intranasally to adult volunteers (Table 1). Both viruses were found to be satisfactorily attenuated for volunteers. The few upper respiratory tract illnesses observed with the recombinant viruses were milder and of shorter duration than those observed with wild-type virus, i.e., a mean of 2.1 days for the attenuated viruses versus 3.5 days for wild-type virus. Only one of 26 volunteers infected with the *ts*-1A2 recombinant developed a mild febrile illness (maximum temperature, 39°C) which lasted for 12 h. None of the 41 volunteers infected with CR-35 virus became febrile.

The human 50% infectious dose (HID_{50}) was approximately $10^{5.0}$ $TCID_{50}$ for both recombinants. An HAI antibody response was detected in only approximately one-half of inoculated volunteers, whereas each of the six volunteers administered wild-type virus developed a serum HAI antibody response. An NI antibody response was detected in two of six volunteers infected with wild-type virus and in less than 5% of individuals given either recombinant. Pre- and postvaccination sera from those vaccinees who failed to develop an HAI antibody response were tested by ELISA to determine whether this more sensitive assay could detect evidence of infection in additional volunteers. A significant number of additional infections were detected by ELISA. For example, each of the 13 volunteers who were given 300 HID_{50} ($10^{7.5}$ $TCID_{50}$) of the CR-35 virus and who failed to develop a serum HAI antibody response developed a serum antibody response detectable by ELISA. Thus, 100% of these volunteers appeared to have been infected with CR-35 virus. Similarly, additional infections were detectable by ELISA among recipients of 10 to 32 HID_{50} ($10^{6.0}$ to $10^{6.5}$ $TCID_{50}$) of the *ts*-1A2 recombinant, and this increased the estimate of proportion of volunteers infected from 38 to 73%. Finally, antigenicity of the CR-35 at a level of 10 to 100 HID_{50} ($10^{6.0}$ to $10^{7.0}$ $TCID_{50}$) appeared to be similar to that of the *ts*-1A2 recombinant in approximately the same dose range, 63 versus 73%.

A greater percentage of CR-35 than of *ts*-1A2 vaccinees shed virus. Vaccinees infected with the CR-35 virus shed virus in slightly higher titer and for a longer duration than did *ts*-1A2 vaccinees. The magnitude and duration of virus shedding was much less for both groups of vaccinees than for volunteers infected with wild-type virus. This observation is consistent with the greatly reduced virulence of these recombinants. Virus shed by both groups of vaccinees retained the phenotypic properties of the respective recombinant. Each of 62 isolates from the CR-35 vaccinees retained the *ts* and *ca* phenotypes. Similarly, each of 19 isolates from the *ts*-1A2 vaccinees were *ts*. To examine in more detail the *ts* phenotype of the virus shed by the vaccinees, isolates from those vaccinees who shed virus for 6 or more days were tested for plaque formation at 34, 37, 38, and 39°C on MDCK monolayer cultures (Tables 2 and 3). The virus shed by the CR-35 vaccinees generally retained the 37 to 38°C shutoff temperature of the vaccine virus, with an occasional isolate exhibiting a shutoff temperature of 39°C (Table 2). Virus shed by the two *ts*-1A2 vaccinees (Table 3) retained the 37°C shutoff temperature.

Lack of transmission of recombinants. No evidence for transmission of CR-35 or *ts*-1A2 recombinants to susceptible controls was observed. Four doubly seronegative volunteers were housed with ten CR-35 vaccinees ($10^{7.5}$ $TCID_{50}$), but virological or immunological evidence of spread of virus was not observed. Similarly, four susceptible controls were housed with 19 *ts*-1A2 vaccinees ($10^{6.0}$ or $10^{6.5}$ $TCID_{50}$), 14 of whom were infected. Similarly, no evidence for transmission of the *ts*-1A2 recombinant to the contacts was obtained.

DISCUSSION

The reappearance of the H1N1 virus in 1977, 20 years after it had last circulated in humans, provided a unique opportunity to evaluate H1N1

TABLE 1. *Response of seronegative volunteers to A/HK/123/77 (H1N1)-ts1A2 recombinant, ca recombinant (CR-35), or wild-type virus*

A/HK/123/77 virus administered	Dose ($TCID_{50}$)	No. tested	% Infected[a]	Virus shedding			Serum HAI antibody[b]			% with serum HAI and/or ELISA antibody response[c]	% with indicated illness			
				% Shedding	Avg duration (days ± SE)[d]	Peak mean $\log_{10}$ titer (±SE)[d,e]	Reciprocal of mean $\log_2$ titer (±SE)		% with ≥4-fold rise in antibody		Febrile (≥37.8°C)	Systemic symptoms (afebrile)	Upper respiratory tract	Any illness
							Pre	Post						
ts-1A2	$10^{6.5}$	23	70	26	0.6 ± 0.3	0.7 ± 0.1	2.0 ± 0.1	2.9 ± 0.3	30	70	4	0	9	13
ts-1A2	$10^{6.0}$	11	82	18	0.9 ± 0.8	0.9 ± 0.4	1.8 ± 0.2	3.4 ± 0.3	55	82	0	0	0	0
ts-1A2	$10^{5.0}$	7	57	28	1.3 ± 0.8	1.3 ± 0.5	1.0 ± 0.0	2.0 ± 0.4	43	57	0	0	14	14
CR-35	$10^{7.5}$	24	100	58	1.6 ± 0.4	1.1 ± 0.2	2.0 ± 0.2	3.4 ± 0.2	45	100	0	0	13	13
CR-35	$10^{7.0}$	5	60	60	5.7 ± 2.4	2.6 ± 1.0	2.2 ± 0.4	3.6 ± 0.7	40	60	0	0	0	0
CR-35	$10^{6.0}$	11	82	45	1.9 ± 0.9	1.5 ± 0.5	2.0 ± 0.2	2.8 ± 0.4	18	64	0	0	0	0
CR-35	$10^{5.0}$	10	50	40	1.6 ± 0.6	1.5 ± 0.3	1.5 ± 0.2	2.4 ± 0.4	30	50	0	0	0	0
Wild type	$10^{4.2}$	6	100	100	5.8 ± 0.2	6.3 ± 0.3	2.0 ± 0.0	6.2 ± 0.4	100	100	67	16	67	83

[a] Evidence of virus shedding and/or a rise in serum HAI or ELISA antibody.
[b] HAI antibodies were tested with A/USSR/77 (H1N1) split vaccine as antigen.
[c] All serum pairs were tested for HAI antibody. Serum pairs from individuals without an HAI rise were tested in ELISA with goat anti-human IgG antiserum, and those pairs from individuals who did not develop a response by HAI or IgG ELISA were tested in ELISA with goat anti-human IgM.
[d] Data from only those volunteers infected were used for calculations. Each vaccinee was tested for 7 days, and volunteers who received wild-type virus were tested for 10 days. SE, standard error.
[e] The amount of virus in the nasal wash specimen from each volunteer was determined, and the peak titers from the volunteers who shed virus were averaged.

recombinant influenza A vaccine viruses in doubly seronegative young adults (3). Two H1N1 recombinant vaccine viruses deriving attenuating genes from the A/Udorn/72-*ts*-1A2 virus or from the A/AA/6/60 *ca* virus were found to be satisfactorily attenuated in the volunteers. Febrile or systemic illness was not observed among 41 volunteers infected with the CR-35 virus, and only 1 of 26 volunteers infected with the *ts*-1A2 recombinant developed a mild, transient febrile response. In contrast, wild-type virus induced febrile or systemic illness in five of six volunteers that lasted from 48 to 96 h. CR-35 and *ts*-1A2 recombinants induced upper respiratory symptoms less often than wild-type virus, and when such symptoms occurred they were less severe than those seen in association with wild-type virus infection. No evidence for transmission of CR-35 or *ts*-1A2 vaccine virus to controls was observed.

The virus shed by vaccinees retained the *ts* or *ca* phenotype, or both. This occurred despite replication of the recombinant viruses for up to 9 days. In contrast, it had previously been found that 25% of doubly seronegative children infected with the A/HK/68-*ts*-1[E] virus shed vi-

TABLE 2. *Characterization of isolates*[a] *from volunteers who received A/HK/123/77 ca recombinant (CR-35) virus for level of temperature sensitivity and presence of the ca phenotype*

Test	Volunteer no.	Day after virus administration	Log_{10} reduction of virus titer at indicated restrictive temp from titer at permissive temp[b]			Shutoff temp (°C)[c]	*ca* phenotype[d]
			37°C	38°C	39°C		
A	1	1	3.1	>3.7	−3.7	37	+
		2	1.7	>3.3	>3.3	38	+
		5	1.8	>3.3	>3.3	38	+
		6	1.5	>3.9	>3.9	38	+
		7	1.8	>4.2	>4.2	38	+
		9	2.1	>4.1	>4.1	37	+
A	2	1	2.4	>3.3	>3.3	37	+
		2	3.3	>3.8	>3.8	37	+
		3	>3.8	>3.8	>3.8	37	+
		8	>4.6	>4.6	>4.6	37	+
A	3	1	>3.6	>3.6	>3.6	37	+
		2	>3.9	>3.9	>3.9	37	+
		3	0.8	1.8	>3.9	39	+
		5	3.1	>3.8	>3.8	37	+
		6	2.7	>3.6	>3.6	37	+
		7	3.0	>3.6	>3.7	37	+
B	4	1	1.6	>4.0	>4.0	38	+
		3	0.5	>3.6	>3.6	38	+
		4	0.1	>4.6	>4.6	38	+
		5	1.2	>4.8	>4.8	38	+
		6	0.8	>4.8	>4.8	38	+
		7	1.0	>4.8	>4.8	38	+
B	5	1	1.5	>4.1	>4.1	38	+
		2	0.8	>4.4	>4.4	38	Not tested
		3	0.2	>4.2	>4.2	38	+
		4	1.6	>4.4	>4.4	38	+
		5	1.5	>4.4	>4.4	38	+
		6	0.8	>4.8	>4.8	38	+
C	6	1	1.0	2.8	>3.8	38	+
		2	1.8	>4.6	>4.6	38	+
		3	1.2	1.8	>5.5	39	+
		4	2.5	>3.8	>2.8	37	+
		6	0.6	>2.0	>4.0	38	+
Virus controls							
A, A/HK/127/77 *ca* virus (CR-35)	A/HK/123/77		>4.9	>4.9	>4.9	37	+
B, A/HK/127/77 *ca* virus (CR-35)	A/HK/123/77		1.7	>4.6	>4.6	38	+
C, A/HK/127/77 *ca* virus (CR-35)	A/HK/123/77		3.2	>3.2	>3.2	37	+
A, A/HK/23/77 wild-type virus	A/HK/123/77		0.1	0.6	1.2	>39	0
B, A/HK/23/77 wild-type virus	A/HK/123/77		0.0	0.2	1.2	>39	0
C, A/HK/23/77 wild-type virus	A/HK/123/77		0.3	0.4	0.6	>39	0

[a] Virus was isolated from nasal wash in MDCK cells and passaged once more in this cell line.

[b] Expressed as plaque-forming units per milliliter.

[c] Defined as the lowest temperature at which a 100-fold reduction of plaque formation occurs in MDCK monolayer cultures compared with the titer at the permissive temperature (34°C).

[d] Defined as the ability of a virus to plaque with equal efficiency in primary chick kidney monolayer cultures incubated at 25 and 34°C.

TABLE 3. *Temperature sensitivity of isolates from volunteers who received A/HK/123/77-ts-1A2 recombinant virus*

Volunteer no.	Day after virus administration	Log_{10} reduction of virus titer at indicated restrictive temp from titer at permissive temp[a]			Shutoff temp (°C)[b]
		37°C	38°C	39°C	
1	1	>2.6	>2.6	>2.6	37
	2	>4.0	>4.0	>4.0	37
	3	>4.3	>4.3	>4.3	37
	4	>3.4	>3.4	>3.4	37
	5	2.8	>4.7	>4.7	37
	6	3.0	>4.5	>4.5	37
	7	>3.0	>3.0	>3.0	37
2	2	4.4	>5.3	>5.3	37
	3	4.4	>5.3	>5.3	37
	5	3.9	>4.5	>4.7	37
	7	>4.0	>4.0	>4.0	37
Virus controls					
HK/77-*ts*-1A2		3.5	>4.8	>4.8	37
HK/77 wild type		0.2	0.3	1.0	>39

[a, b] See Table 2.

rus that lost the *ts* phenotype (25). This ts^+ virus emerged during the late period of virus shedding (25). In the present study, the recombinant viruses that were shed, in general, retained the same level of temperature sensitivity as the input virus, indicating a high degree of genetic stability of this property. In no instance did a ts^+ virus emerge, and only two isolates from CR-35 vaccinees showed evidence of shift in its *ts* property, i.e., 38 to 39°C shutoff. The significance of such a shift is unknown, but a *ca* A/Alaska/6/77 (H3N2) recombinant with a 39°C shutoff has been shown to be attenuated for humans (Murphy et al., unpublished observations).

The HID_{50} for each virus was approximately $10^{5.0}$ $TCID_{50}$. At 300 HID_{50}, the CR-35 virus infected each of 24 volunteers. Only 45% of these volunteers had a rise in HAI antibody titer, but each manifested a seroresponse demonstrable by the HAI or ELISA. The proportion of volunteers infected by 10 to 32 HID_{50} of the *ts*-1A2 recombinant increased from 38 to 73% when the results of the ELISA were combined with those of the HAI test. A similar increase in serological recognition was seen for volunteers given 10 to 100 HID_{50} of the CR-35 virus: 25 to 63%. The low frequency of serum HAI antibody response observed with the H1N1 recombinants was significantly less than that seen previously with *ca* and *ts*-1A2 recombinants prepared from H3N2 wild-type viruses (2, 11-13). In light of the low HAI response, it will be important to evaluate the resistance induced by the CR-35 and *ts*-1A2 H1N1 recombinants by using homologous wild-type H1N1 virus challenge. The significance of the antibodies detected by the ELISA remains unknown.

If *ts* mutations are to be used for the construction of live influenza A virus vaccines, it is essential that such *ts* mutations regularly confer the properties of attenuation, genetic stability, and immunogenicity upon any new epidemic or pandemic virus into which they are transferred by genetic reassortment. The two *ts*-1A2 *ts* genes have regularly conferred a satisfactory level of attenuation on two H3N2 viruses and one H1N1 virus in susceptible adults or children (11, 12; P. Wright, unpublished observations). However, emergence of virus that had lost the *ts* phenotype during infection of a young vaccinee with the A/Alaska/77-*ts*-1A2 recombinant indicates that the goal of complete genetic stability in this highly permissive host has not been met (17). Of interest, the A/Victoria/75-*ts*-1A2 (H3N2) recombinant and, in the present study, the A/HK/123/77-*ts*-1A2 (H1N1) recombinant did not undergo a change in the *ts* phenotype similar to that seen with the A/Alaska/77-*ts*-1A2 recombinant (Wright, personal communication). The basis for the differences in the genetic stability of these three *ts*-1A2 recombinants is unclear.

The genes of the *ca* A/AA/6/60 virus that confer the *ca* and *ts* phenotype have not been completely defined, and it is not known whether these mutations are responsible for attenuation. Since the *ca* donor virus was passaged over 30 times in chick kidney tissue, it is possible that this donor virus bears host range or other non-*ca*, non-*ts* mutations that contribute to the attenuation of the virus and its recombinants (14). Irrespective of the identification of the genes

that confer attenuation, it is clear that A/AA/6/60 *ca* parent and its recombinant viruses are attenuated for lower animals and humans (2, 8, 9, 13). Previously, the level of attenuation of the A/AA/6/60 *ca* H2N2 parent virus could not be fully determined in humans because the virus was tested in volunteers who had experience with virus(es) possessing H2N2 antigens (6). The present study provided information bearing upon this issue in that the CR-35 *ca* recombinant virus, which possessed all non-glycoprotein genes from the A/AA/6/60 parent, was satisfactorily attenuated in doubly seronegative adults. P. Wright and colleagues (unpublished observations) have demonstrated that the CR-35 virus is also satisfactorily attenuated in doubly seronegative children. These results indicate that the genes present in this *ca* donor virus can render an epidemic influenza A virus satisfactorily attenuated for fully susceptible individuals. However, some variation in the *ts* phenotype of other *ca* recombinant viruses derived from the A/AA/6/60 *ca* donor virus has been observed, as well as some variation in their level of attenuation (9, 14, 23). In addition, viruses with altered *ca* phenotype have been isolated from vaccinees who received an H3N2 *ca* recombinant (13). It is not known whether these alterations in phenotypic markers are associated with changes in the level of attenuation of the recombinants. Additional recombinants prepared from other H3N2 and H1N1 wild-type viruses should be evaluated in seronegative adults and the most permissive host, the doubly seronegative child, in order to identify those genetic properties of *ca* recombinant viruses that are reproducibly associated with attenuation, genetic stability, and immunogenicity.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service contracts NO1 AI 42553, NO1 AI 22503, NO1 AI 42528, and NO1 AI 72521 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Cox, N. J., H. F. Maassab, and A. P. Kendal.** 1979. Comparative studies of wild-type and cold-mutant (temperature-sensitive) influenza viruses: nonrandom reassortment of genes during preparation of live virus vaccine candidates by recombination at 25° between recent H3N2 and H1N1 epidemic strains and cold-adapted A/Ann Arbor/60. Virology **97**:190–194.
2. **Davenport, F. M., A. V. Hennessy, H. F. Maassab, E. Minuse, L. C. Clark, G. D. Abrams, and J. R. Mitchell.** 1977. Pilot studies on recombinant cold-adapted live type A and B influenza virus vaccines. J. Infect. Dis. **136**:17–25.
3. **Gregg, M. B., A. R. Hinman, and R. B. Graven.** 1978. The Russian flu—its history and implications for this year's influenza season. J. Am. Med. Assoc. **17**:2260–2263.
4. **Hrabar, A., I. Vodopija, F. E. Andre, J. R. Mitchell, H. F. Maassab, A. V. Hennessy, and F. M. Davenport.** 1977. A placebo-controlled dose-response study of the reactogenicity and immunogenicity of a cold-adapted recombinant A/Victoria/3/75 (H3N2) live influenza virus candidate vaccine in healthy volunteers. Dev. Biol. Stand. **39**:53–60.
5. **Isaacs, A., A. W. Gledhill, and C. H. Andrews.** 1952. Influenza A viruses: laboratory studies with special reference to European outbreaks of 1950–55. Bull. W. H. O. **6**:287–315.
6. **Kitayama, T., Y. Yoga, R. B. Hornick, and W. T. Friedwald.** 1973. Low-temperature-adapted influenza A2/AA/6/60 virus vaccine in man. Infect. Immun. **7**: 119–122.
7. **Maassab, H. F.** 1967. Adaptation and growth characteristics of influenza virus at 25°C. Nature (London) **213**: 612–614.
8. **Maassab, H. F., T. Francis, Jr., F. M. Davenport, A. V. Hennessy, E. Minuse, and G. Anderson.** 1969. Laboratory and clinical characteristics of attenuated strains of influenza virus. Bull. W. H. O. **41**:589–594.
9. **Maassab, H. F., S. B. Spring, A. P. Kendal, and A. S. Monto.** 1978. Biologic characteristics of influenza virus recombinants derived at suboptimal temperatures, p. 721–732. *In* B. W. J. Mahy and R. D. Barry (ed.), Negative strand viruses and the host cell. Academic Press, Inc., New York.
10. **Massicot, J. G., B. R. Murphy, F. Thierry, L. J. Markoff, K. Y. Huang, and R. M. Chanock.** 1980. Temperature-sensitive mutants of influenza virus. Identification of the loci of the 2 *ts* lesions in the Udorn/72-*ts*-1A2 donor virus and the correlation of the presence of these 2 *ts* lesions with a predictable level of attenuation. Virology **101**:242–249.
11. **Murphy, B. R., R. M. Chanock, R. G. Douglas, R. F. Betts, D. H. Waterman, H. P. Holley, Jr., D. L. Hoover, S. Suwanagool, D. R. Nalin, and M. M. Levin.** 1979. Temperature-sensitive mutants of influenza A virus: evaluation of the Alaska/77-*ts*-1A2 temperature-sensitive recombinant virus in seronegative adult volunteers. Arch. Virol., in press.
12. **Murphy, B. R., R. M. Chanock, M. M. Levine, G. A. Van Blerk, E. J. Berquist, R. G. Douglas, Jr., R. F. Betts, R. B. Couch, and T. R. Cate, Jr.** 1979. Temperature-sensitive mutants of influenza A virus: evaluation of the A/Victoria/75-*ts*-1A2 temperature-sensitive recombinant virus in seronegative adult volunteers. Infect. Immun. **23**:249–252.
13. **Murphy, B. R., H. P. Holley, Jr., E. J. Berquist, M. M. Levine, S. B. Spring, H. F. Maassab, A. P. Kendal, and R. M. Chanock.** 1979. Cold-adapted variants of influenza A virus: evaluation in adult seronegative volunteers of A/Scotland/840/74 and A/Victoria/3/75 cold-adapted recombinants derived from the cold-adapted A/Ann Arbor/6/60 strain. Infect. Immun. **23**: 253–259.
14. **Murphy, B. R., L. J. Markoff, R. M. Chanock, S. B. Spring, H. F. Maassab, A. P. Kendal, N. J. Cox, M. M. Levine, R. G. Douglas, Jr., R. F. Betts, R. B. Couch, and T. R. Cate, Jr.** 1980. Genetic approaches to attenuation of influenza A viruses for man. Phil. Trans. R. Soc. Lond. **B288**:401–415.
15. **Murphy, B. R., L. J. Markoff, N. T. Hosier, H. M. Rusten, R. M. Chanock, A. P. Kendal, R. G. Douglas, Jr., R. F. Betts, T. R. Cate, Jr., R. B. Couch, M. M. Levine, D. H. Waterman, and H. P. Holley, Jr.** 1978. Temperature-sensitive mutants of influenza A virus: evaluation of A/Victoria/3/75-*ts*-1[E] recombinant viruses in volunteers. Infect. Immun. **20**:671–677.
16. **Murphy, B. R., E. L. Tierney, B. A. Barbour, R. H. Yolken, D. W. Alling, H. P. Holly, Jr., R. F. Mayner, and R. M. Chanock.** 1980. Use of the enzyme-linked immunosorbent assay to detect serum antibody responses by volunteers who received attenuated influenza A virus vaccines. Infect. Immun. **29**:342–347.

17. **Murphy, B. R., M. D. Tolpin, J. G. Massicot, H. W. Kim, R. H. Parrott, and R. M. Chanock.** 1980. Escape of a highly defective influenza A virus mutant from its *ts* phenotype by extragenic suppression and other types of mutation. Ann. N.Y. Acad. Sci., in press.
18. **Murphy, B. R., F. T. Wood, Jr., J. G. Massicot, S. B. Spring, and R. M. Chanock.** 1978. Temperature-sensitive mutants of influenza virus. XV. The genetic and biological characterization of a recombinant influenza virus containing two *ts* lesions produced by mating two complementing, single lesion *ts* mutants. Virology **88:** 231-243.
19. **Murphy, B. R., F. T. Wood, Jr., J. G. Massicot, and R. M. Chanock.** 1978. Temperature-sensitive mutants of influenza virus. XVI. Transfer of the two *ts* lesions present in the Udorn/72-*ts*-1A2 donor virus to the Victoria/3/75 wild type virus. Virology **88:**244-251.
20. **Murphy, B. R., F. T. Wood, Jr., J. G. Massicot, and R. M. Chanock.** 1979. Temperature-sensitive mutants of influenza A virus. Transfer of the two *ts*-1A2 *ts* lesions present in the Udorn/72-*ts*-1A2 donor virus to the influenza A/Alaska/6/77 (H3N2) wild type virus. Arch. Virol., in press.
21. **Richman, D. D., B. R. Murphy, R. M. Chanock, J. M. Gwaltney, R. G. Douglas, Jr., R. F. Betts, N. R. Blacklow, F. B. Rose, T. A. Parrino, M. M. Levine, and E. S. Caplan.** 1976. Temperature-sensitive mutants of influenza A virus. XII. Safety, antigenicity, transmissibility, and efficacy of influenza A/Udorn/72-*ts*-1[E] recombinant viruses in human adults. J. Infect. Dis. **134:**585-594.
22. **Spring, S. B., H. F. Maassab, A. P. Kendal, B. R. Murphy, and R. M. Chanock.** 1977. Cold-adapted variants of influenza A virus. I. Comparison of the genetic properties of *ts* mutants and five cold-adapted variants of influenza A virus. Virology **77:**337-343.
23. **Spring, S. B., H. F. Maassab, A. P. Kendal, B. R. Murphy, and R. M. Chanock.** 1978. Cold-adapted variants of influenza A virus. II. Comparison of the genetic and biological properties of *ts* mutants and 4 recombinants of the cold-adapted A/AA/6/60 strain. Virol. **55:**233-246.
24. **Spring, S. B., S. R. Nusinoff, J. Mills, D. D. Richman, E. L. Tierney, B. R. Murphy, and R. M. Chanock.** 1975. Temperature-sensitive mutants of influenza virus. VI. Transfer of *ts* lesions from the Asian subtype of influenza A virus (H2N2). Virology **66:**522-532.
25. **Wright, P. F., S. H. Sell, T. Shinozaki, J. Thompson, and D. T. Karzon.** 1975. Safety and antigenicity of influenza A/Hong Kong/68-*ts*-1[E] (H3N2) vaccine in young seronegative children. J. Pediatr. **87:**1109-1116.