INFECTION AND IMMUNITY, Feb. 1979, p. 253-259
0019-9567/79/0023-0253/07$02.00/0

Vol. 23, No. 2

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

BRIAN R. MURPHY,[1]* H. PRESTON HOLLEY, JR.,[2] ERICK J. BERQUIST,[2] MYRON M. LEVINE,[2] SUSAN B. SPRING,[1] HUNIEN F. MAASSAB,[3] ALAN P. KENDAL,[4] AND ROBERT M. CHANOCK[1]

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014[1]; Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland 21201[2]; Department of Epidemiology, Virus Laboratory, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104[3]; and Respiratory Virus Section, Center for Disease Control, Atlanta, Georgia 30333[4]

Received for publication 31 August 1978

Influenza A/Scotland/74 (H3N2) and A/Victoria/75 (H3N2) cold-adapted (*ca*) recombinant viruses, prepared by mating the A/Ann Arbor/6/60 (H2N2) *ca* donor virus and influenza A wild-type virus, were evaluated in adult seronegative volunteers (serum hemagglutination-inhibiting antibody titer, ≤1:8) for level of attenuation, antigenicity, and genetic stability of the temperature-sensitive and *ca* phenotypes. At $10^{7.0}$ to $10^{7.5}$ 50% tissue culture infective doses the A/Scotland/74 and A/Victoria/75 *ca* recombinant viruses were clearly attenuated and antigenic. However, one of eight vaccinees infected with $10^{7.5}$ 50% tissue culture infective doses of the A/Scotland/74 *ca* recombinant had a febrile reaction (39°C). At a 10-fold higher dose ($10^{8.5}$ 50% tissue culture infective doses), 4 of 12 A/Scotland/74 vaccinees had a febrile and/or systemic reaction. Febrile reactions were not observed in volunteers who received the A/Victoria/75 *ca* recombinant virus, whereas 3 of the 12 vaccinees had mild upper respiratory tract symptoms, in one instance associated with mild systemic manifestations. Significantly, the serum hemagglutination- and neuraminidase-inhibiting antibody responses were comparable to those induced by wild-type virus. Both *ca* recombinant viruses were shed in low titer for a short period of time. Each isolate retained the temperature-sensitive phenotype. However, there was evidence of genetic instability of the *ca* marker in that 7 of 24 isolates exhibited some loss of the *ca* property, and one isolate completely lost the capacity to produce plaques at 25°C. The retention of a low level of residual reactogenicity in the A/Scotland/74 *ca* recombinant suggests that acquisition of the *ca* and temperature-sensitive phenotypes by a *ca* recombinant virus may not always bring about a satisfactory level of attenuation for individuals lacking hemagglutinin immunity.

The selection of mutant viruses that replicate efficiently at suboptimal temperatures has yielded viruses that are attenuated for animals and humans (2, 4, 8, 10-14) and, for this reason, the use of cold-adapted (*ca*) mutants of influenza A virus as vaccine strains for humans has been proposed (8). Initially, *ca* mutants of influenza A viruses were produced by successive passage at progressively lower temperature or by plaque selection after abrupt shift of incubation temperature from 33 to 25°C (10, 13). Alternative techniques for producing a vaccine strain after the emergence of a new epidemic or pandemic strain were sought for two reasons: (i) the time needed to produce a *ca* mutant virus of a new variant is at least 3 months, which is too long an interval in the face of an influenza virus epidemic (13); and (ii) *ca* mutants generated by this technique are heterogeneous with respect to in vitro properties (23) and to the level of attenuation for humans (2, 4, 8, 13, 25). Therefore, this approach could not be relied upon to rapidly produce a suitably attenuated vaccine strain of a new variant.

Recently, attenuation of new strains of influenza A virus was accomplished rapidly, i.e. within 6 weeks, by the transfer of attenuating genes (via genetic reassortment) from an attenuated donor virus to a new epidemic variant (19). The A/Ann Arbor/6/60 (A/AA/6/60)

(H2N2) *ca* mutant, which is also temperature sensitive (*ts*) and attenuated for ferrets (14), has been used as such a donor virus. The level of attenuation of this donor strain could not be determined accurately in humans because of the high background of H2 hemagglutinin immunity when the virus was tested in the late 1960s (8). Therefore, *ca* recombinant viruses within the H3N2 subtype were produced to determine whether the A/AA/6/60 donor virus could repeatedly confer an acceptable level of attenuation on each new emerging variant. A/Queensland/6/72 (H3N2) and A/Victoria/3/75 (A/Vic/3/75) (H3N2) *ca* recombinants derived from the A/AA/6/60 *ca* parent have been evaluated for their level of attenuation in humans (3, 5). Since these studies were done in open populations in which the majority of the volunteers were seropositive (serum hemagglutination-inhibiting [HI] antibody titer, $\geq$1:8) at the time of virus administration, the level of attenuation of these recombinants for susceptible individuals could not be fully assessed. This report describes an evaluation of A/Scotland/840/74 (A/Scot/840/74) and A/Vic/3/75 *ca* recombinants (derived from the A/AA/6/60 *ca* parent) for attenuation and antigenicity in adult volunteers with serum HI antibody titers of $\leq$1:8.

MATERIALS AND METHODS

Viruses. The A/AA/6/60 (H2N2) virus was isolated from a throat washing of a patient with influenza-like illness by using primary chick kidney cultures incubated at 36°C. A *ca* mutant was prepared by serially passaging the virus in chick kidney monolayer cultures at reduced temperature of incubation stepwise down to 25°C (36°C, one passage; 33°C, seven passages; 30°C, seven passages; 25°C, seven passages). The virus was then cloned seven times by plaque isolation at 25°C in primary chick kidney tissue. After cloning, the virus was passaged twice in the allantoic cavity of specific pathogen-free eggs at 25°C, and the second passage allantoic fluid suspension was used to prepare the recombinants described below. This A/AA/6/60 *ca* mutant was restricted significantly in plaque formation at 37°C (shutoff temperature, 37°C) but produced plaques efficiently at 25°C (10, 23). The wild-type A/AA/6/60 virus from which this *ca* mutant was derived, as well as the A/Scot/74 and A/Vic/75 wild-type viruses, failed to produce plaques at 25°C in chick kidney monolayers and were not *ts* (shutoff temperature, >39°C).

The A/Scot/840/74 (H3N2) wild-type virus used to produce a *ca* recombinant had four passages in the allantoic cavity of embryonated eggs. The A/Vic/3/75 (H3N2) wild-type virus used to produce a *ca* recombinant had five passages in the allantoic cavity of eggs. The A/Vic/3/75 (H3N2) wild-type virus used to infect volunteers was isolated in specific pathogen-free eggs (SPAFAS, Inc., Norwich, Conn.) and had one additional passage in specific pathogen-free eggs (18).

Recombinant virus containing the hemagglutinin of the A/Equine 1/Prague/56 (Heq 1 Neq 1) virus and the neuraminidase of the A/Georgia/74 (H3N2) virus or the A/Vic/75 (H3N2) virus was used to measure the serum neuraminidase-inhibiting (NI) antibody response of volunteers who received the A/Scot/74 or A/Vic/75 virus, respectively (1, 22).

Preparation of recombinants. The A/Scot/74 *ca* recombinant virus (designated CR-18, clone 7) was produced by coinfection of primary chick kidney cultures with A/Scot/74 wild-type and the A/AA/6/60 *ca* mutant at a multiplicity of infection of 3 for each virus (6). After 48 h of incubation at 25°C, fluid and cells of the doubly infected cell cultures were harvested. This harvest was treated with specific hyperimmune ferret antiserum to the A/AA/6/60 *ca* mutant and then passaged in chick kidney tissue cultures at 25°C; two passages were performed in the presence of immune serum, and one was performed without serum. Virus was then passaged twice in embryonated eggs at 25°C without immune serum. Primary chick kidney monolayer cultures were inoculated with this material, and 10 isolated plaques were picked and subpassaged in the allantoic cavity of embryonated eggs at 25°C. A total of $10^{4.0}$ 50% tissue culture infectious doses ($TCID_{50}$) of clone 7 were inoculated into the allantoic cavity of specific pathogen-free eggs and incubated for 48 h at 32°C. This material, which titrated $10^{8.5}$ $TCID_{50}$ per ml in primary rhesus monkey kidney culture, was shown to be free of adventitious agents as previously described and was used for administration to volunteers (9, 16). This virus produced large plaques with high efficiency at 25°C in chick kidney cultures and had a 39°C shutoff temperature for plaque formation in Madin-Darby canine kidney (MDCK) cell cultures.

The production of the A/Vic/3/75 *ca* recombinant (CR-19) was similar to that of the A/Scot/74 *ca* recombinant, except that the CR-19 underwent two successive plaque passages. The vaccine virus was grown in the allantoic cavity of specific pathogen-free eggs and safety tested as previously described (3). The virus titrated $10^{8.0}$ $TCID_{50}$ per ml. When tested in the same experiment, both CR-18 clone 7 and CR-19 had a 39°C shutoff temperature for plaque formation; there was a $10^{4.5}$-fold reduction of plaque formation in MDCK cultures at 39°C compared with 33°C, but less than a $10^{2.0}$-fold reduction at 38°C. In contrast, the A/Scot/74 and A/Vic/75 wild-type viruses produced plaques with high efficiency at 39°C. Like their A/AA/6/60 parent, both *ca* recombinants failed to complement our prototype group 1 *ts* mutant (24). In addition, both CR-18 clone 7 and CR-19 had the hemagglutinin and neuraminidase glycoproteins of the wild-type parent.

Clinical studies. The wild-type and recombinant *ca* viruses were evaluated at the Clinical Research Center for Vaccine Development of the University of Maryland, Baltimore, Md., in facilities and volunteer populations previously described (18). Informed consent was obtained before participation in the study. The methods used for isolation of volunteers, administration of virus, observation of clinical responses, and collection and processing of serum and nasopharyngeal washes have been described previously (18). Nasopharyngeal specimens were inoculated into primary

rhesus monkey kidney cultures which were incubated at 33°C. The volunteers were selected on the basis of good health and serum HI antibody titers of ≤1:8. This level of serum antibody does not necessarily indicate that the volunteers lacked experience with H3N2 antigens, but it did increase the probability that residual virulence in the vaccine strains would be detected.

Assessment of viral shedding and immune responses. The techniques for measurement of infectivity, for characterization of viral isolates, and for determination of HI, NI, and neutralizing antibody titers have been described previously (18).

The A/Scot/74 *ca* recombinant and A/Vic/75 wild-type viruses were used in the HI test because these antigens were more sensitive for the detection of a serum antibody response than were the recombinants containing the homologous hemagglutinin and the Neq 1 neuraminidase. Whether this increased sensitivity of the H3N2 antigen was due to synergism with N2 antibody or to a property of the hemagglutinin antigen itself was not determined. By using hemagglutinin-specific recombinants, 16 of the 22 serum HI antibody responses were confirmed. These observations indicate that the HI test with an H3N2 virus detected, for the most part, hemagglutinin-specific responses. A recombinant containing the H3 (Vic/75) hemagglutinin and the Neq 1 neuraminidase, adapted to growth in rhesus monkey kidney culture, was used in the neutralization assay to measure nasal wash antibodies in specimens from volunteers who received the A/Vic/3/75 wild-type or A/Vic/3/75 *ca* recombinant (CR-19). The A/Scot/74 vaccine virus was used in the neutralization test because a hemagglutinin-specific recombinant adapted to rhesus monkey kidney tissue was not available. Neutralization assays and infectivity titrations were performed on rhesus monkey kidney tissue grown on Costar 24-well plastic tissue culture plates (Flow Laboratories, Rockville, Md.).

RESULTS

Response of volunteers to the A/Scot/74 and A/Vic/75 *ca* recombinant viruses. The A/Scot/74 *ca* recombinant virus was administered intranasally (1.0 ml per volunteer) at a dose of $10^{8.5}$ $TCID_{50}$ to 12 seronegative volunteers (Table 1). Each volunteer was infected, and four individuals developed a febrile or systemic response (myalgia, chills, sweats, headache, or malaise) that began within the first 48 h after virus administration and lasted for less than 24 h. One of these volunteers had an accompanying rhinitis. Virus was shed by 9 of the 12 volunteers in low titer for a short period. Of the 12 volunteers 10 developed a significant serum HI antibody titer response, and 8 had a significant rise in serum NI antibody titer. Although a wild-type A/Scot/74 virus was not studied for comparative purposes, the magnitude of the antibody response to the high dose of the A/Scot/74 *ca* recombinant was as high, if not higher, than that stimulated by other H3N2 wild-type viruses administered intranasally (16, 19, 21).

A 10-fold lower dose ($10^{7.5}$ $TCID_{50}$ per volunteer) of the A/Scot/74 *ca* virus was administered to 10 volunteers, and 8 were infected; i.e., they shed virus and/or had an immunological response. One volunteer developed a febrile response (maximum temperature, 39°C) which began 1 day after virus administration, lasted for 28 h, and was accompanied by malaise, chills, and headache. Five of the ten volunteers shed virus in low titer for a short period. Four of the eight infected volunteers developed a serum HI antibody response, and five had a rise in serum NI antibody titer.

The five volunteers who had a febrile response or systemic reaction after receiving the A/Scot/74 *ca* recombinant virus had a lower mean pre-inoculation serum NI antibody titer (mean, 1:2.6) than the other infected, but asymptomatic, vaccinees (mean, 1:8). Although this difference is not statistically significant, it is consistent with previous observations that pre-inoculation serum NI antibody is associated with resistance to attenuated as well as wild-type influenza A virus (7, 17, 18).

The A/Vic/75 *ca* recombinant (CR-19) virus was attenuated compared with wild-type virus (Table 1). Of 12 infected vaccinees, 3 developed symptoms; 2 had mild rhinitis (1 with a cough), and 1 had rhinitis associated with malaise, myalgias, and headache. In contrast, 8 of 12 volunteers infected with wild-type A/Vic/75 virus became ill; in 5 instances febrile and/or systemic reactions were observed. The CR-19 virus was shed by seven vaccinees in low titer and for a short period. The immunological response induced by vaccine virus was comparable to that which developed after infection with the wild-type virus.

Characterization of isolates for the *ca* and *ts* phenotypes. A total of 18 isolates obtained from vaccinees infected with the A/Scot/74 *ca* recombinant maintained the *ts* phenotype. Eleven of the isolates were also *ca*. Six exhibited an intermediate degree of cold adaptation which has been termed the "cold-adapted property" (24). Virus with the cold-adapted property produced plaques at 25°C, but the plaque titer at this temperature was more than 100-fold reduced compared with the titer at 33°C and/or the plaques appeared late at 25°C and did not grow appreciably in size during incubation (24). One of the isolates was not cold-adapted at all; i.e., it failed to produce any plaques at 25°C. Each of the six isolates from the A/Vic/75 *ca* vaccinees tested also retained the *ts* property; five were *ca*, and one exhibited the cold-adapted property.

TABLE 1. *Responses of serum HI antibody-negative volunteers to A/Scot/74 and A/Vic/75 ca recombinants*

Virus administered	Dose ($TCID_{50}$)	No. of volunteers	No. infected	Virus shedding			Serum HI antibody response[a]			Serum NI antibody response			Nasal wash neutralizing antibody response (no. with fourfold or greater rise)	No. with:			
							Titer (reciprocal mean log_2)		No. with fourfold or greater rise	Titer (reciprocal mean log_2)		No. with 1.5 (log_2) or greater rise		Fever (temp, ≥37.8°C)	Systemic illness without fever[c]	Upper respiratory tract symptoms[d]	Any illness
				No. shedding	Avg duration (days)[b]	Peak mean log_{10} titer[b]	Pre-inoculation[b]	Post-inoculation[b]		Pre-inoculation[b]	Post-inoculation[b]						
A/Scot/840/74 *ca* recombinant (CR-18)	$10^{8.5}$	12	12	9	1.0 ± 0.25[e]	0.8 ± 0.25	1.9 ± 0.19	5.6 ± 0.55	10[f]	2.6 ± 0.46	5.2 ± 0.58	8[f]	7[f]	3	1	1	4
A/Scot/840/74 *ca* recombinant (CR-18)	$10^{7.5}$	10	8	5	1.4 ± 0.53	0.91 ± 0.21	1.4 ± 0.26	4.0 ± 0.63	4	2.9 ± 0.52	4.6 ± 0.41	5	1	1	0	0	1
A/Vic/3/75 *ca* recombinant (CR-19)	$10^{7.0}$	13	12	7	1.0 ± 0.41	0.71 ± 0.14	2.2 ± 0.21	4.0 ± 0.25	8	<0.5[g]	0.8 ± 0.31	5	5	0	1	3	3
A/Vic/3/75 wild type	$10^{5.2}$	12	12	12	5.0 ± 0.39	3.4 ± 0.47	2.0 ± 0.47	3.8 ± 0.56	6	<0.5	1.1 ± 0.23	5	3[h]	3	2	8	8

[a] The response was measured by using homologous H3N2 antigens.

[b] Determined by using data from infected volunteers only.

[c] A vaccinee was considered to have a systemic illness if myalgias or chills and sweats developed.

[d] Upper respiratory tract illness is defined as an illness observed by two physicians on 2 consecutive days that consists of either or both of the following: (i) pharyngitis (the occurrence of pharyngeal erythema and discomfort) and/or (ii) rhinitis (the development of rhinorrhea).

[e] Mean ± standard error.

[f] Only 11 volunteers were tested.

[g] Titers below 0 could not be accurately estimated, and thus the standard error could not be determined.

[h] Only five volunteers were tested.

DISCUSSION

At $10^{7.0}$ to $10^{7.5}$ $TCID_{50}$ the A/Scot/74 and A/Vic/75 *ca* recombinant viruses were clearly attenuated and antigenic for adult seronegative volunteers (serum HI titer, ≤1:8). However, one of eight vaccinees infected with $10^{7.5}$ $TCID_{50}$ of the A/Scot/74 *ca* recombinant had a febrile reaction. At a 10-fold-higher dose ($10^{8.5}$ $TCID_{50}$) 4 of 12 A/Scot/74 vaccinees had a febrile and/or systemic reaction. Febrile reactions were not observed in volunteers who received the A/Vic/75 *ca* recombinant virus, whereas 3 of the 12 vaccinees had mild upper respiratory tract symptoms, in one instance associated with systemic manifestations. Significantly, the serum HI and NI antibody responses were comparable to those induced by wild-type virus. A more complete interpretation of the reactogenicity of the *ca* recombinant vaccine viruses would require a full evaluation of dose-response relationships such that the reactogenicity of the two viruses could be compared at 100 50% human infectious doses. In the absence of this information, we are left with the suggestion that the A/Scot/74 *ca* recombinant is more reactogenic than the A/Vic/75 *ca* recombinant.

It is of interest that the mild influenzal symptoms observed with the *ca* recombinants were similar to those caused by a comparable dose of an A/Udorn/72 *ts* recombinant virus that also had a 39°C shutoff temperature (21). The retention of this level of residual reactogenicity in the A/Scot/74 *ca* recombinant suggests that acquisition of the *ca* and *ts* phenotypes by a *ca* recombinant virus may not be sufficient in itself to insure a satisfactory level of attenuation for individuals lacking hemagglutinin immunity.

Previous evaluation of the A/AA/6/60 parent and its A/Queensland/72 CR-6 *ca* and *ts* recombinant viruses suggested that these viruses were satisfactorily attenuated for adult volunteers (3, 8). There are several possible explanations for the difference between the previous observations and those of the present investigation. *ca* recombinant viruses derived from the A/AA/6/60 *ca* parent virus differ in their level of temperature sensitivity of plaque formation (24). The A/Queensland/72 CR-6 *ca* recombinant virus has a 37°C shutoff temperature of plaque formation like its *ca* parent virus, whereas the A/Dunnedin/73 CR-13 *ca* recombinant virus is not *ts* (24). The A/Scot/74 CR-18 and A/Vic/75 CR-19 *ca* recombinant viruses are intermediate in their level of temperature sensitivity (shutoff temperature, 39°C); thus they are less *ts* than their A/AA/6/60 parent virus (37°C) but more *ts* than their wild-type parent virus (>39°C). Observations, summarized elsewhere, implicate the *ts* phenotype and, more specifically, the level of temperature sensitivity as one determinant of attenuation of viruses for animals or humans (20). Thus, the ability of the CR-18 and CR-19 *ca* recombinant viruses to cause febrile and/or systemic illness could reflect this partial loss of temperature sensitivity, i.e., a 39°C shutoff temperature for the recombinants versus 37°C for the A/AA/6/60 *ca* parent.

Another explanation for the incomplete attenuation of the CR-18 and CR-19 viruses could be that a gene or set of genes other than those that determine the *ca* and *ts* properties also contribute to attenuation of the A/AA/6/60 *ca* parent virus and its recombinants. If such an attenuating gene or genes were not on the ribonucleic acid segments that determine the *ca* and *ts* properties, the level of attenuation and the *ca* and *ts* phenotypes could segregate independently during genetic reassortment. In this case, the level of attenuation of *ca* recombinant viruses would be determined in part by the presence or absence of these hypothetical attenuating genes. Such a non-*ts* attenuating gene has been detected in a 5-flurouracil *ts* mutant of an influenza A virus, and this non-*ts* gene segregated independently of *ts* genes (20). Non-*ts* attenuating genes are presumably present in influenza A viruses attenuated by multiple passages in a heterologous host (15; J. S. Oxford, D. J. McGeoch, G. C. Schild, and A. S. Beare, *in Negative Strand Viruses and the Host Cell*, in press), and the A/AA/6/60 has had 20 passages in primary chick kidney and 2 passages in eggs. A candidate for such a non-*ts* gene would be the gene that codes for the nonstructural protein. The parental origin of the genes in the CR-18 and CR-19 viruses has been determined by polyacrylamide gel electrophoresis of the ribonucleic acid segments of the A/AA/6/60 *ca* parent and the *ca* recombinants (A. P. Kendal and N. J. Cox, unpublished data). The CR-18 received the ribonucleic acid segments 1, 2, and 3 and the genes coding for the nucleoprotein and matrix protein from the A/AA/6/60 parent, whereas the hemagglutinin, neuraminidase, and nonstructural protein genes came from the A/Scot/74 parent virus. The CR-19 *ca* recombinant received each of its genes from the A/AA/6/60 parent except the ribonucleic acid segment 2, hemagglutinin, and neuraminidase genes. If the nonstructural protein gene of the A/AA/6/60 parent contributes significantly to attenuation of this donor virus and its recombinants, its absence from the A/Scot/74 *ca* recombinant could account for the apparent reactogenicity of this *ca* recombinant. More *ca* recombinants must be examined to answer this question.

Another possible explanation for reactogenicity of *ca* recombinant viruses is reversion or

suppression of one or more attenuating genes, resulting in a change to wild-type phenotype. This explanation would not apply if the attenuating genes specify the *ts* phenotype, since all 24 isolates from this study retained the *ts* property. The majority (16 of 24) of the isolates were also *ca*. However, there was evidence for genetic instability of the *ca* marker in that seven isolates had the intermediate cold-adapted property, and one isolate had completely lost the capacity to produce plaques at 25°C. The viruses with altered *ca* phenotype were recovered from both ill and asymptomatic volunteers. The event leading to this genetic alteration could have occurred in the tissue cultures used for virus isolation and not in the volunteer. The low titer of virus present in the nasopharyngeal wash specimens made it possible to examine this issue directly. If genes other than the *ts* and *ca* genes were responsible for attenuation, we lack an in vitro test to identify these genes and to determine whether "reversion" to wild-type phenotype occurred after replication in humans.

The factors responsible for the attenuation of the A/AA/6/60 *ca* donor virus and its recombinants are not completely understood. However, coordinated biological, genetic, and biochemical investigations are underway in an effort to identify the attenuating genes (6, 23, 24).

LITERATURE CITED

1. **Aymard-Henry, M., M. T. Coleman, W. R. Dowdle, W. G. Laver, G. C. Schild, and R. G. Webster.** 1973. Influenza virus neuraminidase and neuraminidase inhibition test procedures. Bull. W.H.O. **48**:199-202.
2. **Beare, A. S., H. F. Maassab, D. A. J. Tyrrell, A. N. Slepuskin, and T. S. Hall.** 1971. A comparative study of attenuated influenza viruses. Bull. W.H.O. **44**: 593-598.
3. **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.
4. **Edwards, E. A., R. E. Mammen, M. J. Rosenbaum, R. O. Peckinpaugh, J. R. Mitchell, H. F. Maassab, E. Minuse, A. V. Hennessy, and F. M. Davenport.** 1973. Live influenza vaccine studies in human volunteers. Symp. Ser. Immunobiol. Stand. **20**:289-294.
5. **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.
6. **Kendal, A. P., N. J. Cox, B. R. Murphy, S. B. Spring, and H. F. Maassab.** 1977. Comparative studies of wild type and "cold-mutant" (temperature-sensitive) influenza viruses: geneology of the matrix (M) and nonstructural (NS) proteins in recombinant cold-adapted H3N2 viruses. J. Gen. Virol. **37**:145-159.
7. **Kim, H. W., J. O. Arrobio, C. D. Brandt, R. H. Parrott, B. R. Murphy, D. D. Richman, and R. M. Chanock.** 1976. Temperature-sensitive mutants of influenza A virus. Response of children to the influenza A/Hong Kong/68-*ts*-1[E] (H3N2) and influenza A/Udorn/72-*ts*-1[E] (H3N2) candidate vaccine viruses and significance of immunity to neuraminidase antigen. Pediatr. Res. **10**:238-242.
8. **Kitayama, T., Y. Yogo, 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.
9. **Knight, V.** 1964. The use of volunteers in medical virology. Prog. Med. Virol. **6**:1-26.
10. **Maassab, H. F.** 1967. Adaptation and growth characteristics of influenza virus at 25°C. Nature (London) **213**: 612-614.
11. **Maassab, H. F.** 1969. Biologic and immunologic characteristics of cold-adapted influenza virus. J. Immunol. **102**:728-732.
12. **Maassab, H. F.** 1975. Properties of influenza virus 'cold' recombinants, p. 755-763. *In* B. W. J. Mahy and R. D. Barry (ed.), Negative strand viruses, vol. 2. Academic Press Inc., London.
13. **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.
14. **Maassab, H. F., A. P. Kendal, and F. M. Davenport.** 1972. Hybrid formation of influenza virus at 25°C. Proc. Soc. Exp. Biol. Med. **139**:768-773.
15. **McMahon, D., and G. C. Schild.** 1972. Segregation of antigenic and biological characteristics during influenza virus recombination. J. Gen. Virol. **15**:73-77.
16. **Murphy, B. R., E. G. Chalhub, S. R. Nusinoff, and R. M. Chanock.** 1972. Temperature-sensitive mutants of influenza virus. II. Attenuation of *ts* recombinants for man. J. Infect. Dis. **126**:170-178.
17. **Murphy, B. R., J. A. Kasel, and R. M. Chanock.** 1972. Association of serum anti-neuraminidase antibody with resistance to influenza in man. N. Engl. J. Med. **286**: 1329-1332.
18. **Murphy, B. R., L. J. Markoff, N. T. Hosier, H. M. Rusten, R. M. Chanock, A. P. Kendal, R. G. Douglas, 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.
19. **Richman, D. D., B. R. Murphy, R. B. Belshe, H. M. Rusten, R. M. Chanock, N. R. Blacklow, T. A. Parrino, F. B. Rose, M. M. Levine, and E. S. Caplan.** 1977. Temperature-sensitive mutants of influenza A virus. XIV. Production and evaluation in volunteers of influenza A/Georgia/74-*ts*-1[E] recombinant temperature-sensitive viruses. J. Infect. Dis. **136**:256-262.
20. **Richman, D. D., B. R. Murphy, and R. M. Chanock.** 1977. Demonstration of a non-temperature sensitive growth restricting mutation in a *ts* mutant of influenza A virus: implications for live virus vaccine development. Virology **83**:356-364.
21. **Richman, D. D., B. R. Murphy, R. M. Chanock, J. M. Gwaltney, R. G. Douglas, 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. **Richman, D. D., B. R. Murphy, W. L. Cline, and D. W. Alling.** 1976. Determination of influenza virus neuraminidase inhibition titers. Bull. W.H.O. **52**:233-234.
23. **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.

24. **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. Arch. Virol. **55**:233–246.
25. **Van Kirk, J. E., J. Mills, Jr., and R. M. Chanock.** 1971. Evaluation of low temperature grown influenza A2/Hong Kong virus in volunteers. Proc. Soc. Exp. Biol. Med. **136**:34–41.