VIROLOGY 77, 337-343 (1977)

Cold-Adapted Variants of Influenza Virus A

I. Comparison of the Genetic Properties of *ts* Mutants and Five Cold-Adapted Variants of Influenza Virus A

S. B. SPRING,[1] H. F. MAASSAB,*[,2] A. P. KENDAL,† B. R. MURPHY, AND R. M. CHANOCK

*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014; *Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor, Michigan 48104; and †Respiratory Virology Branch, Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333*

Accepted November 3, 1976

The genetic properties of seven cold-adapted variants of influenza virus A were compared with those of nine 5-fluorouracil (5-FU)-induced *ts* mutants. The 5-FU mutants had previously been placed into seven complementation-recombination groups; five of the seven cold-adapted variants also had the *ts* phenotype, and all five were shown to share the group 1 lesion. Three of the cold variants also had additional *ts* lesions.

INTRODUCTION

Recent attempts to control influenza A virus epidemic and pandemic disease by live virus vaccination have involved the production of updated vaccine strains by transfer of gene(s) conferring attenuation from attenuated donors to the current epidemic wild-type (wt) subtype (Beare and Hall, 1971; Beare and McCahon, 1972; Fenner, 1972; Maassab *et al.*, 1972; McCahon and Schild, 1972; Murphy *et al.*, 1973, 1974; Beare *et al.*, 1975). Three types of donor viruses have been suggested: (i) temperature-sensitive (*ts*) mutants (often derived by mutagenesis) which are restricted in their ability to produce plaques at 38 or 39°; (ii) cold-adapted variants which can replicate equally well at 25 and 33°, while the wt parent can replicate only at 33°; and (iii) viruses attenuated by passage in eggs or tissue culture. Vaccine strains prepared by each of these methods appear to be attenuated both in man and animals when compared with the homologous wild-type parent (Maassab, 1967, 1969, 1970; Boudreault *et al.*, 1968; Maassab *et al.*, 1969, 1972; Mills *et al.*, 1969; Smorodincev, 1969; Beare *et al.*, 1971; Beare and Hall, 1971; Mills and Chanock, 1971; Beare and McCahon, 1972; McCahon and Schild, 1972; Murphy *et al.*, 1972, 1973, 1974, 1976; Edwards *et al.*, 1972; Izuchi and Mizutani, 1973; Mizutani and Izuchi, 1973; Davenport *et al.*, 1975; Beare *et al.*, 1975; Richman *et al.*, 1975; Spring *et al.*, 1975b). However, *ts* mutants and cold variants have the potential advantage that they possess *in vitro* markers which are associated with attenuation. These markers can be assessed in tissue culture before the viruses are evaluated in man.

A set of nine temperature-sensitive mutants of influenza A derived by mutagenesis with 5-fluorouracil (5-FU) has recently been placed into seven complementation groups (Spring *et al.*, 1975b) on the basis of a complementation-recombination assay carried out directly on the tissue culture monolayer. Previous studies (Maassab, 1967; Medvedeva *et al.*, 1969; Maassab, 1969, 1970; Maassab *et al.*, 1969) have suggested that cold-adapted variants of influ-

[1] Address reprint requests to Dr. Spring.

[2] Supported in part by the office of the U.S. Army Medical Research and Development Command, Department of the Army, under Research Contract DADA 17-73-C-3060.

Copyright © 1977 by Academic Press, Inc.
All rights of reproduction in any form reserved.

ISSN 0042-6822

enza virus are temperature sensitive in chick kidney tissue culture. We therefore sought to use the 5 FU mutants as genetic probes to ascertain whether the two sets of viruses shared *ts* lesions.

MATERIALS AND METHODS

Viruses

(i) ts Mutants. The production and characterization of the *ts* mutants and *ts* recombinants used as prototypes for each of the complementation groups have been detailed previously (Murphy *et al.*, 1975; Richman *et al.*, 1975; Spring *et al.*, 1975a,b).

(ii) Cold-adapted variants. Two methods were used in the production of cold variants: (i) The A/AA/Marton/43 (H0N1), A/FM/1/47 (H1N1), and A/AA/6/60 (H2N2) strains were adapted by multiple successive passages in chick kidney tissue culture at progressively lower temperatures (intervals of 3°) (Maassab, 1967). (ii) The A/AA/2/65 (H2N2), A/AA/2/67 (H2N2), A/Aichi/2/68 (H3N2), and A/AA/1/70 (H3N2) strains were cold-adapted by a second method: The parental strain was passaged three times in chick kidney tissue culture at 25°; plaques were then selected at 25° in this same cell line (Maassab, 1970). The properties of these viruses are summarized in Table 1.

Antigenic Analysis

The subtype of the hemagglutinin antigen was determined by hemagglutination inhibition (HI) using A/AA/Marton/43 (H0N1), A/Great Lakes/389/1965 (H2N2), and A/HK/X-31/68 (H3N2) as reference antigens. Reference antisera were produced in chickens to the A/AA/Marton/43 (H0N1), A2/Taiwan/1/64 (H2N2), and A/Aichi/2/68 (H3N2) strains.

Procedures similar to the WHO test protocol for neuraminidase specificity were employed (Aymard-Henry *et al.*, 1973). Antiserum to the N1 antigen was prepared in chickens using the H equi 1 N1 (Bel) recombinant as antigen; antiserum to the N2 antigen was prepared using the H equi 1 N2 (Aichi) recombinant as antigen.

Growth and Assay of Infectious Virus

Suspensions of *ts* mutants or cold variants were prepared by allantoic inoculation of 10-day-old embryonated eggs and harvest of the allantoic fluid after 48–72 hr of incubation at 33°. Embryonated eggs were obtained from either Truslow Farms (Chestertown, Maryland) or SPAFAS Inc. (Storrs, Connecticut).

Plaque assays were performed using rhesus monkey kidney (RMK) monolayers grown on plastic petri dishes. An agar overlay consisting of 0.9% agarose, medium L-15, and antibiotics was used (Mills and Chanock, 1971; Murphy *et al.*, 1974). In studies performed at restrictive temperatures (37, 38, and 39°), the tissue culture plates were sealed in steel boxes which were placed in a constant-temperature circulating water bath (maximum variation, 0.05°). Tissue cultures were purchased from Flow Laboratories, Inc. (Rockville, Maryland).

Studies comparing the efficiency of plating of virus strains and isolates at 25 or 33° were carried out in chick kidney (CK) cultures. The preparation of these cells from 1- to 4-day-old chicks and the assay procedure have been previously described (Maassab, 1967, 1969). The cells in such cultures are predominantly epitheloid, but fibroblast-like cells are also in evidence.

Complementation–Recombination Assay

The technique for the detection of complementation–recombination on the assay plate has been described in detail previously (Mills and Chanock, 1971; Spring *et al.*, 1975b) and is summarized in Results.

RESULTS

Antigenic Analysis

Evaluation by HI and neuraminidase inhibition (NI) indicated that each of the cold variants had the surface antigens of the wild-type parent (Table 1).

Characterization of Cold-Adapted Variants with Respect to Efficiency of Plaque Formation (EOP)

Replicate sets of CK monolayers were inoculated with serial dilutions of cold var-

iants or parental wt strains and incubated for 3-5 days at 25 or 33°. The titer in plaque-forming units (PFU) per milliliter at each temperature was then calculated. Table 2 presents the log reduction of the titer at 25° with respect to the titer at 33°. These data indicate that each of the cold variants produced plaques with high efficiency at 25°, whereas the wt parent of each variant did not induce plaques at this temperature.

Replicate sets of RMK monolayers were inoculated with serial dilutions of the cold variants or parental wt strains and incubated for 3 days at 37, 38, or 39° or for 5 days at 33°; the titer in PFU per milliliter at each temperature was then calculated. Table 2 presents the log reduction of the

TABLE 1

ts LESIONS IN COLD VARIANTS

Cold variant	Method of derivation[a]	Shared lesions (complementation group)[b]	Shutoff temperature[c]	Antigenic subtype[d]
A/AA/Marton/43-H0N1	Stepwise	–	>39	$H0_{43}N1_{43}$
A/FM/1/47-H1N1	Stepwise	–	>39	$H1_{47}N1_{47}$
A/AA/6/60-H2N2	Stepwise	1	37	$H2_{60}N2_{60}$
A/AA/2/65-H2N2	Plaque selection	1	38	$H2_{65}N2_{65}$
A/AA/2/67-H2N2	Plaque selection	1, 3, 5	37	$H2_{67}N2_{67}$
A/Aichi/2/68-H3N2	Plaque selection	1, 6	38	$H3_{68}N2_{68}$
A/AA/1/70-H3N2	Plaque selection	1, 3, 6	38	$H3_{70}N2_{70}$

[a] Reviewed in Maassab, 1970; summarized in Materials and Methods.

[b] Determined by complementation-recombination assay on RMK monolayers using 5-FU-Hong Kong-*ts* mutants and recombinants as prototypes.

[c] Taken as temperature at which there is a 100-fold or greater loss in plaquing efficiency on RMK monolayers.

[d] Determination described in text.

TABLE 2

PLAQUING EFFICIENCY OF COLD-ADAPTED VARIANTS AND PARENTAL STRAINS AT PERMISSIVE AND RESTRICTIVE TEMPERATURES

Virus	Log reduction in plaque titer at 25° from titer observed at 33° in CK tissue culture	Log reduction of plaque titer at indicated restrictive temperature from titer observed at permissive temperature (33°) in RMK tissue culture			Shutoff temperature in RMK cells[a] (°C)
		37°	38°	39°	
Cold variants					
A/AA/Marton/43-H0N1	0.2	N.D.[b]	N.D.	1.0	>39
A/FM/1/47-H1N1	0.6	N.D.	N.D.	1.0	>39
A/AA/6/60-H2N2	−0.1	>5.9	>5.9	>5.9	37
A/AA/2/65-H2N2	1.2	0.3	>4.9	>4.9	38
A/AA/2/67-H2N2	0.2	2.8	>5.7	>6.5	37
A/Aichi/2/68-H3N2	0.2	1.8	>5.8	>5.8	38
A/AA/1/70-H3N2	0.5	1.0	>5.9	>5.9	38
Parental strains					
A/AA/Marton/43-H0N1	>6.7	0.0	0.0	0.0	>39
A/FM/1/47-H1N1	>6.9	N.D.	N.D.	0.0	>39
A/AA/6/60-H2N2	>8.3	0.0	0.0	0.0	>39
A/AA/2/65-H2N2	>6.8	N.D.	N.D.	1.0	>39
A/AA/2/67-H2N2	>6.9	N.D.	N.D.	0.0	>39
A/Aichi/2/68-H3N2	>7.3	N.D.	N.D.	0.0	>39
A/AA/1/70-H3N2	>6.9	N.D.	N.D.	0.0	>39

[a] Defined as a 100-fold or greater reduction in plaquing efficiency on RMK monolayers.

[b] Not done.

titer at each of the three restrictive temperatures with respect to the titer at the permissive temperature (33°). The shutoff temperature of each mutant was arbitrarily chosen as the lowest temperature at which there was a 100-fold or greater decrease in titer. The values shown in Table 2 represent the average log reduction of two or three tests on different lots of RMK cells. None of the parental viruses was restricted significantly in plaquing efficiency at 39°. Only the A/AA/2/65 strain produced fewer plaques at 39°, and, in this instance, the restriction was only 10-fold. In contrast, five of the seven cold variants were temperature sensitive and sufficiently restricted in plaque formation at 39° to permit genetic analysis with the prototype 5-FU *ts* mutants. Two of the cold variants were not sufficiently temperature sensitive to be analyzed by complementation–recombination with the prototype 5-FU *ts* mutants.

Genetic Characterization of Cold-Adapted Variants

The five *ts* cold-adapted variants were analyzed by the plate complementation–recombination technique to determine in which complementation group or groups they belonged. The nine 5-FU mutants previously assigned to seven complementation groups were used as prototype strains for this purpose (Spring *et al.*, 1975b).

Pairs of mutants were mixed and incubated at 4° for 18 hr. Serial dilutions of the mixtures were made and inoculated onto RMK monolayer cultures. The cultures were then incubated at 39°. Replicate monolayers were also inoculated with each individual mutant and incubated at 33° or 39°. The resulting titer at 33° was used to estimate the input multiplicity. Few if any plaques developed following incubation of singly infected cultures at 39°; in those instances in which a mutant produced a small number of plaques at 39°, the appropriate adjustment was made in calculating the number of plaques expected following dual infection with other mutants at restrictive temperature. In each test, one or more wild-type strains were assayed at 33° and 39°, and the titers for each virus were always approximately equal at the two temperatures.

When dually infected cell monolayers were incubated at 39°, the number of plaques which developed varied from none for certain pairs of mutants to a quantity which equalled or exceeded the number expected assuming a Poisson distribution of dually infected cells and an efficiency of plaque formation by such cells of 100%. Mutant pairs such as A/AA/Marton/6/60-cold variant and *ts* 463 for which the ratio of observed to expected plaques was greater than 1 (Table 3) were assumed to possess *ts* lesions in different cistrons of their genome. Thus, each of the viruses could supply the biochemical function which was defective in the other, resulting in complementation followed by reassortment of the viral genomes. Mutant pairs which failed to produce plaques at the restrictive temperature, e.g., R1 and A/AA/Marton/6/60-cold variant, were assumed to have *ts* lesions in the same cistron of their genome. The results summarized in Table 3 represent at least four tests for each mutant pair. The data suggested that all five *ts* cold-adapted viruses shared the *ts* lesion represented by complementation group 1. In addition, the cold variants derived from the 1967, 1968, and 1970 strains appeared to possess additional lesions. The findings are summarized in Tables 1 and 3. Three pairs of viruses exhibited variable interaction (Table 3). The values presented are consistent with the results of 70% of multiple assays (10–15 assays per pair). Variability of genetic interaction between certain pairs of mutants has been observed previously in the complementation–recombination assay, and several hypotheses have been proposed to explain it (Spring *et al.*, 1975a).

DISCUSSION

The data presented in Tables 1 and 3 suggest that the five cold variants which are temperature sensitive share the group 1 lesion. The concurrent acquisition of a *ts* mutation during selection for cold adaptation has also been observed with mutants of poliovirus, Japanese B encephalitis vi-

TABLE 3

PRODUCTION OF PLAQUES AT RESTRICTIVE TEMPERATURE FOLLOWING DUAL INFECTION WITH PUTATIVE SINGLE-LESION *ts* MUTANTS AND COLD VARIANTS[a]

Cold-adapted variant	Complementation group and prototype 5-FU *ts* viruses[b]								
	1	2	3	4	5		6		7
	R1	R8	2C	304	315	422	454	464	463
A/AA/6/60-H2N2	<0.001	50	5	250	2	6	20	20	100
A/AA/2/65-H2N2	<0.001	4	2	20	5	33	3	1	20
A/AA/2/67-H2N2	<0.003	1	<0.001[c]	6	<0.01	<0.01	1	1	20
A/Aichi/2/68-H3N2	<0.002	1[c]	10	2	10	2	<0.001	<0.01	10
A/AA/1/70-H3N2	<0.01	5	<0.001[c]	10	1	1	<0.01	<0.002	10

[a] Values are based on Poisson distribution, assuming that it is necessary for a cell to receive 1 plaque-forming unit of each virus to produce a plaque at 39°. The formula $(1 - e^{mA})(1 - e^{mB})$(number of cells) is used where mA and mB are the input multiplicities of the infecting viruses. Values are representative of a minimum of four tests between each pair. Each test was carried out on a separate lot of RMK cells, and calculations of input virus were based on the titer at 33° of each mutant on that lot of cells.

[b] Spring *et al.* (1975b).

[c] Variable interaction was exhibited in these crosses. From 10 to 15 tests were carried out for these pairs, and the values presented are consistent with 70% of the tests.

rus, and measles virus (Dubes and Chapin, 1956; Dubes and Wenner, 1957; Hammon *et al.*, 1963; Hozinski *et al.*, 1966). Since the five cold variants of influenza were independently derived, it appears that the process of low-temperature selection may preferentially select viruses with *ts* lesions on the complementation group 1 portion of the genome. Spontaneous or mutagen-induced mutants of influenza A (Hirst, 1973; Sugiura *et al.*, 1972, 1975) or mutagen-induced mutants of fowl plaque virus (Markushin and Ghendon, 1973; Scholtissek and Bowles, 1975) often appear to occur preferentially in one or two complementation groups. Preliminary findings suggest that the group 1 lesion may be associated with a defect in cRNA synthesis and probably with the transcriptase complex.[3] It is likely that the first obstacle to replication of a negative-strand virus would be the synthesis of cRNA which functions as mRNA and thus starts the synthesis of viral products. If the group 1 function is an enzyme, it is possible that certain mutations result in altered enzyme conformation and, consequently, a shift in the temperature optimum such that the enzyme activity would be both cold adapted and *ts*, whereas other alterations in the amino acid sequence may induce only cold adaptation.

However, these findings do not preclude more than one cistron being involved in the cold adaptation property: (i) There are two cold variants, A/AA/Marton/43 (H0N1) and A/FM/1/47 (H1N1), which are cold-adapted and not *ts*. (ii). Additional *ts* lesions are also present in three cold variants, A/AA/2/57 (H2N2), A/Aichi/2/68 (H3N2), and A/AA/1/70 (H3N2). It is noteworthy that the group 5 and 6 lesions in these viruses also appear to involve cRNA and vRNA, respectively.[3] Studies are currently in progress to segregate by recombination the groups 1, 3, 5, and 6 *ts* lesions occurring in multilesioned cold variants and to ascertain whether single lesion *ts* segregants retain the cold-adaptation property.

[3] These data are based on complementation–recombination studies (Spring and Chanock, manuscript in preparation) between the 5-Fu Hong Kong *ts* prototypes and the WSN *ts* mutants which have been studied biochemically by Sugiura *et al.* (1975) and by Krug *et al.* (1975).

ACKNOWLEDGMENTS

The expert technical assistance of Mrs. Helen Lester and Mr. W. Lee Cline is greatly appreciated.

REFERENCES

AYMARD-HENRY, M., COLEMAN, M. T., DOWDLE, W. R., LAVER, W. G., SCHILD, G. C., and WEBSTER, R. G. (1973). Influenza virus neuraminidase and

neuraminidase-inhibition test procedures. *Bull. WHO* **48**, 199–202.

BEARE, A. S., and HALL, T. S. (1971). Recombinant influenza A viruses as live vaccines for man. *Lancet* **2**, 1271–1273.

BEARE, A. S., MAASSAB, H. F., TYRRELL, D. A. J., SLEPUSKIN, A. N., and HALL, T. S. (1971). A comparative study of attenuated influenza viruses. *Bull. WHO* **44**, 593–598.

BEARE, A. S., and MCCAHON, D. (1972). Virulence and infectivity of influenza A viruses in relation to surface antigens. *In* "International Symposium on Influenza Vaccines for Men and Horses" (F. T. Perkins and R. H. Regamey, eds.), Symposium Series in Immunobiological Standards Vol. 20, pp. 144–151. S. Karger, Basel.

BEARE, A. S., SCHILD, G. C., and CRAIG, J. W. (1975). Trials in man with live recombinants made from A/PR/8/34 (H0N1) and wild H3N2 influenza viruses. *Lancet* **1**, 729–732.

BOUDREAULT, A., LUSSIER, G., and PAVILANIS, V. (1968). Caracteres biologiques de souches du virus de l'influenza adaptees a 29°C et a 41°C. *Canad. J. Microbiol.* **14**, 867–874.

DAVENPORT, F. M., HENNESSY, A. V., MINUSE, E., MAASSAB, H. F., ANDERSON, G. R., MITCHEL, J. R., HEFFELFINGER, J. C., and BARRET, C. D., JR. (1975). Pilot studies on mono and bivalent live attenuated influenza virus vaccines. *In* "Proceedings. Symposium on Live Influenza Vaccine," pp. 105–113. Yugoslavian Academy of Science and Arts, Zagreb.

DUBES, G. R., and CHAPIN, M. (1956). Cold adapted genetic variants of polio viruses. *Science* **124**, 586–588.

DUBES, G. R., and WENNER, H. A. (1957). Virulence of polioviruses in relation to variant characteristics distinguishable on cells *in vitro*. *Virology* **4**, 275–296.

EDWARDS, E. A., MAMMEN, R. E., ROSENBAUM, M. J., PECKENPAUGH, R. O., MICHELL, J. R., MAASSAB, H. F., MINUSE, E., HENNESSY, A. V., and DAVENPORT, F. H. (1972). Live influenza vaccine studies in human volunteers. *In* "International Symposium on Influenza Vaccines for Men and Horses" (F. T. Perkins and R. H. Regamey, eds.), Symposium Series in Immunobiological Standards. Vol. 20, pp. 289–294. S. Karger, Basel.

FENNER, F. (1972). The possible use of temperature sensitive conditional lethal mutants for immunization in viral infections. *Advan. Exp. Med.* **31**, 131–144.

HAMMON, W. MCD., ROHITAYODHIN, S., and RHIM, J. S. (1963). Studies on Japanese B encephalitis virus vaccines from tissue culture. IV. Preparation and characterization of pool of attenuated OCT-541 line and/or human vaccine trial. *J. Immunol.* **91**, 295–305.

HIRST, G. K. (1973). Mechanism of recombination. I. Factors influencing recombination rates between temperature sensitive mutants of strain WSN and the classification of mutants into complementation recombination groups. *Virology* **55**, 81–93.

HOZINSKI, V. I., SEIBEL, V. B., PANTELYEVA, N. S., MAZUROVA, S. M., and NOVKOVA, E. A. (1966). The rct_{40} and T_{50} markers and the characteristics of some variants of measles virus. *Acta Virol.* **10**, 20–27.

IZUCHI, T., and MIZUTANI, H. (1973). Studies on live influenza virus vaccine. I. Attenuated influenza A2 Hong Kong virus and its biological and immunological characteristics. *Virology (Tokyo)* **23**, 134–140.

KRUG, R. M., UEDA, M., and PALESE, P. (1975). Temperature sensitive mutants of influenza WSN defective in virus specific RNA synthesis. *J. Virol.* **16**, 790–796.

MAASSAB, H. F. (1967). Adaptation and growth characteristics of influenza virus at 25°C. *Nature (London)* **213**, 612–614.

MAASSAB, H. F. (1969). Biologic and immunologic characteristics of cold adapted influenza virus. *J. Immunol.* **102**, 728–732.

MAASSAB, H. F. (1970). Development of variants of influenza virus. *In* "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 542–566. Academic Press, New York.

MAASSAB, H. F., FRANCES, T., JR., DAVENPORT, F. M., HENNESSEY, A. V., MINUSE, E., and ANDERSON, G. (1969). Laboratory and clinical characteristics of attenuated strains of influenza virus. *Bull. WHO* **41**, 589–594.

MAASSAB, H. F., KENDAL, A. P., and DAVENPORT, F. M. (1972). Hybrid formation of influenza viruses at 25°. *Proc. Soc. Exp. Biol. Med.* **139**, 768–773.

MARKUSHIN, S. G., and GHENDON, YU. Z. (1973). Genetic classification and biological properties of temperature-sensitive mutants of fowl plaque virus. *Acta Virol.* **17**, 369–376.

MCCAHON, D., and SCHILD, G. C. (1972). Segregation of antigenic and biological characteristics during influenza virus recombination. *J. Gen. Virol.* **15**, 73–77.

MEDVEDEVA, T. E., ALEXANDROVA, G. I., and SMORODINSEV, A. A. (1969). Differentiation of temperature sensitive variants of influenza A2 viruses on the basis of plaque formation. *J. Virol.* **2**, 456–460.

MILLS, J., V., VAN KIRK, J., HILL, D. A., and CHANOCK, R. H. (1969). Evaluation of influenza mutants for possible use in a live virus vaccine. *Bull. WHO* **41**, 599–601.

MILLS, J., V., and CHANOCK, R. M. (1971). Temperature-sensitive mutants of influenza virus. I. Behavior in tissue culture and in experimental animals. *J. Infect. Dis.* **123**, 145–157.

MIZUTANI, H., and IZUCHI, T. (1973). Studies on

live influenza vaccine. II. Evaluation of cold adapted influenza A2 Hong Kong in volunteers. *Virology (Tokyo)* 23, 141-147.

MURPHY, B. R., CHALUB, E. G., NUSINOFF, S. R., and CHANOCK, R. M. (1972). Temperature-sensitive mutants of influenza virus. II. Attenuation of *ts* recombinants for man. *J. Infect. Dis.* 126, 170-178.

MURPHY, B. R., CHALHUB, E. G., NUSINOFF, S. P., KASEL, J., and CHANOCK, R. M. (1973). Temperature sensitive mutants of influenza virus. III. Further characterization of the ts-1[E] influenza A recombinant (H3N2) virus in man. *J. Infect. Dis.* 128, 479-487.

MURPHY, B. R., HODES, D. S., NUSINOFF, S. R., SPRING-STEWART, S., TIERNEY, E. L., and CHANOCK, R. M. (1974). Temperature-sensitive mutants of influenza virus. V. Evaluation in man of an additional *ts* recombinant virus with a 39°C shutoff temperature. *J. Infect. Dis.* 130, 144-149.

MURPHY, B. R., SPRING, S. B., and CHANOCK, R. M. (1976). Live vaccine: production and use. *In* "Influenza: virus, vaccines and strategy" (Philip Selby, ed.), Proceedings of a working group on pandemic influenza, Rougemont 26-28, January, pp. 177-197. Academic Press, New York.

RICHMAN, D. D., MURPHY, B. R., SPRING, S. B., COLEMAN, M. T., and CHANOCK, R. M. (1975). Temperature sensitive mutants of influenza virus. IX. Genetic and biological characterization of *ts*-1[E] lesions when transferred to a 1972 (H3N2) influenza A virus. *Virology* 66, 551-565.

SCHOLTISSEK, C., and BOWLES, A. L. (1975). Isolation and characterization temperature-sensitive mutants of fowl plaque virus. *Virology* 67, 576-587.

SMORODINCEV, A. A. (1969). The efficacy of live influenza vaccines. *Bull. WHO* 41, 585-588.

SPRING, S. B., NUSINOFF, S. R., MILLS, J. V., RICHMAN, D. D., TIERNEY, E. L., MURPHY, B. R., and CHANOCK, R. M. (1975a). Temperature-sensitive mutants of influenza. VI. Transfer of *ts* lesions from the Asian subtype of influenza A virus (H2N2) to the Hong Kong subtype (H3N2). *Virology* 66, 522-532.

SPRING, S. B., NUSINOFF, S. B., TIERNEY, E. L., RICHMAN, D. D., MURPHY, B. R., and CHANOCK, R. M. (1975b). Temperature-sensitive mutants of influenza. VIII. Genetic and biological characterization of *ts* mutants of influenza virus A (H3N2) and their assignment to complementation groups. *Virology* 66, 542-550.

SUGIURA, A., TOBITA, K., and KILBOURNE, E. D. (1972). Isolation and preliminary characterization of temperature sensitive mutants of influenza virus. *J. Virol.* 10, 639-647.

SUGIURA, A., UEDA, M., TOBITA, K., and ENOMOTO, C. (1975). Further isolation and characterization of temperature-sensitive mutants of influenza virus. *Virology* 65, 363-373.

VAN KIRK, J. E., MILLS, J., V., and CHANOCK, R. M. (1971). Evaluation of low temperature grown influenza A2/Hong Kong Virus in volunteers. *Proc. Soc. Exp. Biol. Med.* 136, 34-41.