SECTION 4

RECENT PROGRESS IN THE DEVELOPMENT AND ASSESSMENT OF LIVE ATTENUATED VACCINES

Chairman: PROFESSOR F. M. DAVENPORT

Use of temperature-sensitive mutants of influenza A virus as live virus vaccine strains. Evaluation in laboratory animals, adults and children

BRIAN R. MURPHY
B.A., M.D.

SUSAN B. SPRING
B.S., Ph.D.

DOUGLAS D. RICHMAN
B.A., M.D.

ROBERT M. CHANOCK
M.D.

*Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases,
National Institutes of Health, Bethesda, Maryland 20014*

Summary

Temperature-sensitive (ts) recombinants of influenza A virus were evaluated for use in a live virus vaccine. Evidence from several sources suggested that the ts lesions were responsible for attenuation of these mutants. Specification of attenuation by defined genetic lesions which can be assayed for in the laboratory offers an advantage to the use of ts viruses for vaccination. This means that ts recombinants can be assessed for genetic stability during vaccine development, production and later during usage in man. One ts virus, influenza A/Hong Kong/68-ts-1[E], with a 38°C shut-off temperature, had the following properties desirable for a live virus vaccine: (1) satisfactory infectivity for seronegative (serum HI antibody titre $\leq 1:8$) adults; (2) satisfactory attenuation for adults; (3) capacity to stimulate local and serum anti-haemagglutinin and anti-neuraminidase antibodies in seronegative volunteers; (4) stimulation of resistance to virulent, wild type virus; (5) relative genetic stability *in vivo*; (6) lack of communicability in man; (7) replication to high titre in avian leucosis virus-free eggs; and (8) localization of ts lesions to genes that do not code for the haemagglutinin and neuraminidase. The ts lesions of

influenza A/Hong Kong/68-ts-1[E] virus were transferred to more current viruses within the H₃N₂ subtype (influenza A/Udorn/307/72 and influenza A/Georgia/101/74). These recombinant Udorn/72 and Georgia/74 ts viruses, which possessed the same shut-off temperature and the same ts lesions as the influenza A/Hong Kong/68-ts-1[E] parent virus, exhibited a pattern of infection and attenuation in hamsters and man similar to their ts parent. These data suggest that ts mutants which are sufficiently attenuated for man, could serve as donors of ts lesions for the rapid production of an attenuated vaccine when new antigenic variants arise.

When the influenza A/Hong Kong/68-ts-1[E] virus was administered to children who lacked both anti-haemagglutinin and anti-neuraminidase antibody the virus replicated for a longer period than in adults and mild fever developed in some of the young vaccinees. A minority of children shed wild type revertant virus. The emergence of wild type virus in children, but not in adults, probably reflected the more extensive replication of the virus in the doubly seronegative children. The implications of these findings to the development and testing of live influenza A virus vaccines were discussed.

TEMPERATURE-sensitive (ts) mutants have been derived from virtually every major virus group and in

Requests for reprints: Dr Brian R. Murphy, Laboratory of Infectious Diseases, National Institutes of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland 20014, U.S.A.

every instance *ts* mutation has led to a reduction in virulence for the natural or experimental host (Richman *et al.*, 1975). *Ts* mutation can be produced in essentially every cistron of the genome and thus a large array of genetic defects can be assessed for their effect upon the composite property of virulence. Evidence from a number of sources suggests that it is the *ts* lesion (or lesions) which is responsible for the attenuation exhibited by *ts* mutants (Mackenzie, 1969; Mills, Chanock and Alling, 1969; Murphy *et al.*, 1972; Spring *et al.*, 1975a, b; Richman *et al.*, 1975). Specification of attenuation by a defined genetic lesion which can be assayed for in the laboratory offers an advantage to the use of *ts* mutants for vaccination. Thus, *ts* mutants used as vaccine strains can be assessed for genetic stability during vaccine production and later during infection in man. In this manner the genetic basis for attenuation can be monitored directly during all phases of vaccine development, manufacture and usage.

During the past few years, conditional lethal *ts* mutants of influenza A virus have been evaluated for their attenuation in man and for their suitability for use in a live virus vaccine. Mutants which are restricted in their replication *in vitro* at 37–38°C should be similarly limited in their growth in the lower respiratory tract, the major site of significant pathology, which has a temperature of 37°C. However, such mutants should grow with reasonable efficiency in the cooler passages of the upper respiratory tract, which have a temperature of 32–34°C, and thereby stimulate local and systemic immunological defence mechanisms. Furthermore, live influenza A virus vaccines could be produced rapidly by transfer of a *ts* defect (or *ts* defects) from an attenuated *ts* donor virus to a new antigenic variant of this virus (Maassab, 1969; McCahon and Schild, 1972; Spring *et al.*, 1975a). For this approach to succeed it is necessary that the new antigenic variant into which a *ts* gene (or genes) is transferred manifests a level of attenuation and antigenicity similar to its attenuated donor. In this presentation it will be indicated that this is the case.

Production and genetic evaluation of *ts* mutants of influenza A virus

Initially *ts* mutants of wild type influenza A/Great Lakes/1965 (H_2N_2) virus were produced by chemical mutagenesis with 5-fluorouracil (Mills and Chanock, 1971). Two mutants, Great Lakes/65-*ts*-1 and Great Lakes/65-*ts*-2, were isolated and characterized. The *ts* lesions of the Great Lakes/65-*ts* viruses were subsequently transferred via genetic recombination to a wild type influenza A/Hong Kong/1968 (H_3N_2) virus (Table 1) (Spring *et al.*, 1975a). Four clones of Hong Kong/68-*ts* recombinant virus were isolated and characterized genetically (Table 1). The *ts* lesions of

one of these recombinants, the influenza A/Hong Kong/68-*ts*-1[E] virus, were then transferred to the wild type influenza A/Udorn/307/1972 virus (Richman *et al.*, 1975) and later to the wild type influenza A/Georgia/101/1974 virus (Richman *et al.*, unpublished observations). Recombinant clones from each cross were isolated and characterized antigenically and genetically (Table 1).

Seven complementation-recombination groups of influenza A virus have been described independently by Sugiura, Tobita and Kilbourne (1972), Hirst (1973) and Spring *et al.* (1975b). The number and nature of the *ts* lesions present in the recombinant viruses derived from the Great Lakes/65-*ts*-1 and *ts*-2 mutants was determined by mating *ts* recombinants with each other, with their *ts* parent, and with mutants isolated in this laboratory which contained *ts* lesions representative of seven complementation-recombination groups. Two viruses were considered to possess *ts* lesions on the same cistron if coinfection of Rhesus monkey kidney (RMK) monolayer cultures at the restrictive temperature of 39°C failed to produce a significant number of plaques. In contrast, viruses possessing lesions in different cistrons underwent complementation and recombination under these conditions and plaques were produced with high efficiency at restrictive temperature following dual infection of RMK cells. An example of this type of genetic interaction is shown in Table 2. The results of the genetic evaluation of the recombinants derived from the Great Lakes/65-*ts*-1 and *ts*-2 mutants are presented in Table 1. It is of interest that the Udorn/72-*ts*-1[E] recombinant clones 16 and 24 were similar to the Hong Kong/68-*ts*-1[E] parent virus in the number and type of *ts* lesions (two lesions, one in complementation-recombination group 1 and the other in group 2) and shut-off temperature (38°C). The Udorn/72-*ts*-1[E] clone 13 virus, which possessed only the complementation group 1 lesion of the parent virus, exhibited a higher shut-off temperature, i.e., 39°C.

Evidence that the *ts* gene itself specifies attenuation

A study of the replication of the H_2N_2 and H_3N_2 mutants or recombinants of influenza A virus in hamster pulmonary tissue revealed that each of the viruses was restricted in growth in comparison to wild type virus. Since these mutants were either mutagenized directly or were recombinants of mutagenized virus, it was possible that genes other than the *ts* genes were altered by the mutagen and that these non-*ts* genes were responsible for the observed restriction of replication. The following observations from recent studies of *ts* mutants of influenza A virus in animals argues that it is the *ts* lesion(s) itself that specifies attenuation. (1) There was a greater restriction of replication of *ts* viruses in the lungs (37°C) than in the nasal turbinates (34°C). If non-

TABLE 1. History, antigenic phenotype, and genetic characterization of temperature sensitive clones of influenza A virus administered to volunteers

Clone designation	Origin of clone	Antigenic class	Shut-off temperature <i>in vitro</i> (°C)	Number of segments of influenza virus genome with a ts lesion	Complementation-recombination group
Hong Kong/68-ts-1[A]	Recombination of Great Lakes/65-ts-1	H ₃ /68 N ₂ *	37	2	1 and 5
Hong Kong/68-ts-1[E]	(H ₂ /65 N ₂ /65) with wild type Hong Kong/68	H ₃ /68 N ₂ *	38	2	1 and 2
Hong Kong/68-ts-1[H]	(H ₃ /68 N ₂ /68)	H ₃ /68 N ₂ *	39	2	1 and 2
Hong Kong/68-ts-2[C]	Recombination of Great Lakes/65-ts-2 (H ₂ /65 N ₂ /65) with wild type Hong Kong/68 (H ₃ /68 N ₂ /68)	H ₃ /68 N ₂ *	39	1	3
Udorn/72-ts-1[E] 16	Recombination of Hong Kong ts-1[E] (H ₃ /68 N ₂ /68) with wild type Udorn/72 (H ₃ /72 N ₂ /72)	H ₃ /72 N ₂ *	38	2	1 and 2
Udorn/72-ts-1[E] 24	(H ₃ /68 N ₂ /68) with wild type Udorn/72 (H ₃ /72 N ₂ /72)	H ₃ /72 N ₂ *	38	2	1 and 2
Udorn/72-ts-1[E] 13	(H ₃ /72 N ₂ /72)	H ₃ /72 N ₂ /72	39	1	1
Georgia/74-ts-1[E] 2	Recombination of Hong Kong/68-ts-1[E] (H ₃ /68 N ₂ /68) with wild type Georgia/74 (H ₃ /74 N ₂ /74)	H ₃ /74 N ₂ /74	38	2	1 and 2

* It has not been determined whether these clones have the N₂/65 or N₂/68 neuraminidase.

TABLE 2. Example of complementation-recombination between two ts recombinant viruses*

Multiplicity of input of indicated influenza A virus (H ₃ N ₂) recombinant (pfu/cell)†		Average number of plaques per culture incubated at 39°C	
ts-1[E]	ts-2[C]	Expected‡	Observed
1·86	0·28	7400	> 1000
0·186	0·028	74	500
0·0186	0·0028	0·74	24

Note: ts-1[E] or ts-2[C] virus inoculated alone failed to produce plaques at 39°C but titred $2·8 \times 10^6$, and $4·2 \times 10^5$ pfu/ml respectively at 33°C.

* Data previously published, Spring *et al.*, Transfer of ts Lesions from the Asian Subtype of Influenza A Virus (H₂N₂) to the Hong Kong Subtype (H₃N₂). *Virology* (1975), 66, 522.

† Each virus mixture was inoculated on to 60 mm Petri dish monolayer cultures containing $1·5 \times 10^6$ cells per dish.

‡ Based on Poisson distribution, assuming that it is necessary for each cell to receive 1 pfu of each virus to produce a plaque at 39°C. The formula $(1 - e^{-mA})(1 - e^{-mB}) \cdot 100$ is used where *mA* and *mB* are the input multiplicities of the infecting viruses.

lesions were responsible for restriction of replication, then the decrease in viral growth should be equivalent in the lungs and nasal turbinates (Spring *et al.*, 1975b; Richman *et al.*, 1975). (2) The level of restriction of recombinant ts viruses in the lungs was a function of their shut-off temperature (Table 3), i.e. the lower the shut-off temperature the lower the level of replication. (3) Wild type revertants (ts⁺) of

TABLE 3. Suppression of growth in the hamster lung of intranasally administered temperature sensitive mutants of influenza A/Hong Kong/68 or influenza A/Udorn/72 virus

Virus	Shut-off temperature	Reduction of growth in hamster lungs of ts virus compared to homologous wild type virus (log ₁₀ TCID ₅₀ /g)
Hong Kong/68-ts-1[A]	37	4·0*
Hong Kong/68-ts-1[E]	38	2·8
Hong Kong/68-ts-1[H]	39	2·0
Hong Kong/68-ts-2[C]	39	1·8
Udorn/72-ts-1[E] 16	38	2·0†
Udorn/72-ts-1[E] 13	39	0·8

* The level of replication of the Hong Kong/68-ts viruses was determined on pooled lung specimens from five hamsters tested daily for the first 4 days after infection. The maximum level of replication of the ts virus was subtracted from that of wild type virus. The data represent the average reduction from two similar experiments for each virus except ts-1[H].

† The level of replication of the Udorn/72 ts viruses was determined on individual lung specimens (six hamsters/day) for the first 3 days after infection. The average replication of the ts viruses for the 3-day period was subtracted from the average replication of the wild type virus.

the Hong Kong/68-ts-1[E] virus grew as well as the wild type virus in the hamster lung (Richman *et al.*, unpublished observations). If non-ts lesions were responsible for restriction of replication, ts⁺ revertants which possessed the non-ts mutations should have manifested restricted replication in comparison

to the parent wild type virus. (4) Similarly, recombinant ts⁺ clones produced by recombination of the Hong Kong/68-ts-1[E] and Hong Kong/68-ts-2[C] viruses grew to the same level in the hamster lung as wild type virus (Richman *et al.*, unpublished observations). (5) There was a similar level of growth restriction *in vivo* of the Hong Kong/68-ts-1[E] and the genetically equivalent Udorn/72-ts-1[E] double lesion recombinants (clones 16 and 24) despite the fact that the latter viruses were recombinants derived from the former virus and non-ts mutations should not have been conserved during recombination (Richman *et al.*, 1975). Collectively these observations suggest that it was the ts lesion(s) that specified the observed level of growth restriction. Therefore, in the future it would not be unreasonable to expect that viruses that acquire ts lesions via genetic recombination would exhibit a level of growth restriction specified by those ts lesions.

Evaluation of the ts recombinant viruses in adult volunteers

A summary of the response of adult seronegative (serum haemagglutination-inhibition (HI) antibody titre of 1 : 8 or less) volunteers to the 4 Hong Kong/68-ts recombinant viruses is presented in Table 4. The ts-1[A] virus, which had a 37°C shut-off temperature and which exhibited the most restriction of replication in the hamster lung, failed to infect volunteers. Ts-1[H] and ts-2[C], the two ts viruses which had a 39°C shut-off temperature and which were the least restricted in the hamster lung, were attenuated in comparison to wild type virus; however, the two viruses retained the capacity to induce mild febrile influenzal illness. Interestingly the two viruses had the same shut-off temperature and manifested a similar level of clinical reactogenicity although they contained ts lesions affecting different cistrons. This suggested that it was the level of temperature sensitivity (i.e. level of defectiveness) and not a specific biochemical defect which was the determinant of attenuation in man. The Hong Kong/68-ts-1[E] virus, which had a 38°C shut-off temperature and which exhibited an intermediate level of replication in hamsters, produced only a mild cold-like illness in a minority of volunteers, but induced solid resistance to disease produced by a virulent wild type virus challenge. These recombinant viruses had each undergone the same number of passages in bovine kidney culture, but they exhibited a gradient of virulence in man that correlated with the extent of temperature defectiveness imposed by the ts lesion(s). These results, in the context of similar observations in the hamster, supported the suggestion that it was the ts lesions and not other genetic lesions that were primarily responsible for attenuation.

The Hong Kong/68-ts-1[E] virus appeared to exhibit the proper balance between attenuation and immunogenicity desired of a strain to be used for vaccination. This virus had the following properties: (1) capacity to stimulate moderate levels of local and serum anti-haemagglutinin and anti-neuraminidase antibodies (Murphy *et al.*, 1973); (2) genetic stability after replication in seronegative adults and older children (Murphy *et al.*, 1972, 1973; Kim *et al.*, unpublished observation); (3) genetic stability after growth in leucosis virus-free eggs (Murphy *et al.*, 1973); (4) lack of communicability (Murphy *et al.*, 1972; Wright *et al.*, 1975); (5) failure to induce alterations in pulmonary function (Hall *et al.*, unpublished observations) in man; (6) safety and antigenicity in the elderly and chronically ill (Douglas *et al.*, unpublished observations). These characteristics suggested that the Hong Kong/68-ts-1[E] virus might serve as a satisfactory donor of its ts lesions to new antigenic variants of influenza A virus.

Genetic analysis revealed that the Hong Kong/68-ts-1[E] virus possessed ts lesions on two segments of the influenza A virus genome and these RNA segments segregated independently of the genes that coded for the neuraminidase and the haemagglutinin glycoproteins (Murphy *et al.*, 1975). This property of the Hong Kong/68-ts-1[E] virus made it possible to produce recombinants possessing the ts-1[E] genetic lesions and both surface antigens of new, wild type influenza A viruses which emerged following prevalence of the Hong Kong virus. Thus, the ts lesions from the Hong Kong/68-ts-1[E] virus were transferred to the wild type influenza A/Udorn/307/72 (H₃N₂) virus (antigenically indistinguishable from the A/England/42/72 prototype virus) (Richman *et al.*, 1975). Two subsets of virus possessing the Udorn/72 haemagglutinin and the ts phenotype were characterized. The first subset, of which clones 1 and 24 were the prototypes, possessed both ts lesions (complementation groups 1 and 2) and the 38°C shut-off temperature of the Hong Kong/68-ts-1[E] parent (Table 1). Evaluation in hamsters revealed that the parent Hong Kong/68-ts-1[E] virus and the genetically equivalent recombinant Udorn clones 1 and 24 were similar to each other in the following respects: (1) level of replication in lungs and nasal turbinates; (2) low level of reversion to ts⁺ phenotype in the lungs; (3) protection induced to homologous wild type virus challenge. The second subset, of which clone 13 was the prototype, possessed only one of the two ts lesions present in the Hong Kong/68-ts-1[E] donor and had a 39°C shut-off temperature. In hamsters, clone 13 was less stable genetically and was less restricted in replication than clones 1 and 24 which possessed both ts lesions of ts-1[E] virus. In man (Table 5), the single and double lesioned Udorn/72 recombinants exhibited a difference

TABLE 4. Response of seronegative (serum HI antibody titre $\leq 1:8$) adult volunteers to the intranasal administration of a temperature sensitive mutant of wild type influenza A/Hong Kong/68 (H₃N₂) virus*

Virus (Dose TCID ₅₀ /volunteer)	Number of ts lesions	Shut-off temperature <i>in vitro</i> (°C)	Number of volunteers	Average number of days of virus shedding	Virus recovered ts phenotype	% of volunteers showing four-fold or greater rise in serum and/or nasal wash antibody	Clinical response		
							Maximum type of illness observed	Maximum temp (°C)	Protection against wild type viral challenge
Hong Kong/68-ts-1[A] (10 ^{5.5})	2	37	13	0	—	0	None	37	No
Hong Kong/68-ts-1[E] (10 ^{5.6})	2	38	17	1-9	Yes	84%	Mild cold	37	Yes
Hong Kong/68-ts-1[H] (10 ^{5.2})	2	39	8	2-3	No	100%	Mild febrile 'flu	37.5	Not tested
Hong Kong/68-ts-2[C] (10 ^{5.5})	1	39	9	3-0	Yes	89%	Mild febrile 'flu	37.5	Yes
Hong Kong/68-wild type (10 ^{4.5})	0	>40	7	4-3	—	100%	Febrile 'flu	40	Not tested

* Summary data from Murphy *et al. Journal of Infectious Diseases*, 1972, 126, 170 and 1974, 130, 144.TABLE 5. Response of seronegative (serum HI antibody titre $\leq 1:8$) adult volunteers to the intranasal administration of a temperature sensitive mutant or wild type influenza A/Udorn/307/72 (H₃N₂) virus*

Virus (Dose TCID ₅₀ /volunteer)	Number of ts lesions	Shut-off temperature	Number of volunteers	Average number of days of virus shedding	Virus recovered ts phenotype	% of volunteers showing four-fold or greater rise in serum and/or nasal wash antibody	Clinical response	
							Maximum type of illness observed	Maximum temperature (°C)
Udorn/72-ts-1[E] 24 (10 ^{5.2})	2	38	20	1-2	Yes	80	Mild cold	37
Udorn/72-ts-1[E] 16 (10 ^{5.7})	2	38	9	0-7	Yes	78	None	37
Udorn/72-ts-1[E] 13 (10 ^{7.0})	1	39	18	1-4	No	94	Mild febrile 'flu	38
Udorn/72-wild type (10 ^{5.0-10^{4.6}})	0	>40	15	3-8	—	60	Febrile 'flu	38.9

* Summary data from Richman *et al.*, 1975.

virulence (Richman *et al.*, unpublished observations). The Udorn/72 clone 13 (39°C) virus retained the capacity to cause mild febrile influenzal illness, like the Hong Kong/68-ts-1[H] (39°C) and ts-2[C] (39°C) viruses, but was attenuated in comparison to wild type influenza virus. This level of attenuation could not be explained as an effect of tissue culture (TC) passage of the clone 13 virus since in an additional study 10^4 TCID₅₀ of wild type Udorn/72 virus at the same passage level as the clone 13 virus produced febrile influenzal disease which was considerably more severe than the mild influenzal illness caused by 10^7 TCID₅₀ of the clone 13 ts recombinant (Richman *et al.*, unpublished observations).

Two clones of Udorn/72-ts-1[E] virus (Clone 16 and 24) which contained both ts-1[E] lesions were administered to seronegative adult volunteers and were found to be as attenuated and antigenic as the Hong Kong/68-ts-1[E] donor virus. They were satisfactorily attenuated, stable genetically, active antigenically, and they provided protection against a wild type virus challenge. These results provide further support for the concept that the ts lesions confer attenuation, and that viruses that have a specified ts lesion or set of lesions will behave predictably in man. This view gained further support from a limited trial in man of a recent ts-1[E] recombinant. The two ts lesions of ts-1[E] were transferred from the Hong Kong/68-ts-1[E] virus to a wild type influenza A/Georgia/101/1974 virus (Richman *et al.*, unpublished observations). This recombinant ts virus was suitably attenuated and antigenic when given to thirty-two seronegative volunteers. In contrast, the wild type Georgia/74 virus induced febrile influenzal disease. In summary, the two ts-1[E] lesions have been transferred three times to a wild type virus bearing a new haemagglutinin and in each instance the new ts recombinant exhibited a similar level of attenuation and antigenicity for seronegative adults. Thus, it seems likely that transfer of the

ts-1[E] lesions to any new influenza A virus will predictably result in attenuation of the recombinant possessing the new surface antigens.

Evaluation of the Hong Kong/68 and Udorn/72 ts-1[E] viruses in children

Children suffer significant morbidity from influenza A virus infection (Foy, Cooney and McMahan, 1973) and, in addition, have been implicated as important vectors of this infection in the community (Monto *et al.*, 1969; Hall, Cooney and Fox, 1973). A live virus vaccine is needed for this population, especially because inactivated influenza A virus vaccines have been shown to be highly reactogenic and poorly antigenic in very young children (Hennessey and Davenport, 1967, 1974). For these reasons, the Hong Kong/68-ts-1[E] (H₃N₂) candidate live virus vaccine strain, which had previously been shown to be safe and protective in seronegative adult volunteers, was administered intranasally at the Children's Hospital of D.C. to twenty-one children at a dose of 10^5 TCID₅₀ (Table 6) (Kim *et al.*, unpublished observations). One group contained fifteen children (5–11 years of age) who lacked serum antibody to the haemagglutinin ($\leq 1:8$) but possessed serum antibody to the neuraminidase antigen. The second group included six children (28 to 46 months of age) who lacked serum antibody to both haemagglutinin and neuraminidase surface antigens of the influenza A virus, i.e. doubly seronegative children. Twelve of the fifteen children in the first group were infected, but only one child developed mild rhinitis; six of the twelve infected vaccinees shed virus for a short interval, while eleven of the group developed an immunological response. In contrast, each of the six vaccinees who lacked serum antibody for both surface antigens of the virus shed a relatively larger quantity of virus over a longer interval than the first group; in addition four children developed a transient febrile response during infection and

TABLE 6. Response of seronegative (serum HI antibody titre $\leq 1:8$) children to the intranasal administration of influenza A/Hong Kong/68-ts-1[E] virus

Vaccinees with pre-existing serum antineuraminidase antibody (> 1:2)	Dose of virus (TCID ₅₀ per vaccinee)	Number in group	Number shedding virus	Number with ts+ virus in original naso-pharyngeal wash or swab specimens	% with four-fold or greater rise in serum and/or nasal wash antibody	Number with temperature elevation (range in °C)
Yes	$10^{5\dagger}$	15	6	0	80%	0
No	$10^{5\dagger}$	6	6	0	100%	4 (38·2–39·0)
No	$10^{4\dagger}$	18	14	4	88%	4* (38·6–39·4)

* Two additional vaccinees had an elevated temperature but one shed an enterovirus and the other a rhinovirus. Three six contact controls had fever (not associated with influenza A virus infection) of 38·4–39·8°C.

† Summary data from Kim *et al.* (manuscript in preparation).

‡ Summary data from Wright *et al. Pediatric Research* (1975) 9, 347.

three had mild rhinitis. An additional eighteen doubly seronegative children were given 10^4 TCID₅₀ of the Hong Kong/68-ts-1[E] virus at Vanderbilt University and the reactogenicity of the virus was reduced but the virus maintained a high level of antigenicity (Table 6) (Wright *et al.*, 1975). Two children who lacked serum antibody to both surface antigens of influenza virus were given the influenza A/Udorn/72-ts-1[E] recombinant virus, which had the same genetic lesions as the Hong Kong/68-ts-1[E] recombinant virus. Both of the children were infected and one developed a transient fever. None of the twenty-three vaccinees given the Hong Kong/68-ts-1[E] or Udorn/72-ts-1[E] recombinant at Children's Hospital of D.C. developed signs or symptoms of lower respiratory tract involvement.

These findings suggest that anti-neuraminidase immunity naturally acquired from previous infection provided some protection against illness produced by the candidate vaccine virus. Only when doubly seronegative children were evaluated did it become apparent that the ts-1[E] recombinant viruses retained some virulence. It seems likely that a complete assessment of the virulence of candidate live influenza A vaccine viruses can only be made in individuals who lack immunity to both surface antigens of the influenza A virus. It is important to achieve attenuation for such persons especially if one plans to use live attenuated vaccines to control pandemic disease caused by new antigenic variants of influenza A virus. If the next pandemic shift involves the haemagglutinin and neuraminidase antigens, every member of the population might resemble the doubly seronegative child in lacking immunity to both surface antigens. In this circumstance viruses which were acceptably attenuated for individuals with neuraminidase immunity might produce febrile responses similar to those induced by the ts-1[E] recombinants in doubly seronegative children.

The ts-1[E] recombinants were shown recently to contain discrete ts lesions which were located on two different RNA pieces of the segmented genome (Murphy *et al.*, 1975). Despite the existence of ts lesions of two separate RNA segments of the viral genome, the recombinants exhibited evidence of genetic instability during infection of two of eight doubly seronegative young children during the studies at Children's Hospital of D.C. These revertants, which could not be detected in the original nasopharyngeal swab specimen, represented only a very small proportion of the virus population (10^{-2} to 10^{-3}) produced during infection of doubly seronegative children and the emergence of revertant virus under these conditions was not associated with lower respiratory tract disease. However, four of the eighteen children who received 10^4 TCID₅₀ during the studies at Vanderbilt University shed revertant

virus in their original nasopharyngeal wash specimens. In contrast, the ts-1[E] recombinants were stable genetically when isolated from a considerably larger group of seventy-eight adults and six older children who lacked serum HI antibody but possessed neuraminidase antibody. It appeared that emergence of ts⁺ revertants occurred only in the absence of immunity to both haemagglutinin and neuraminidase antigens; presumably only in this circumstance did vaccine virus infection occur without restriction.

In view of the occurrence of reversion and low-grade fever, studies with the ts-1[E] recombinants in children have been suspended. Although the ts-1[E] recombinants did not prove to be the ideal donors of ts lesions for a live influenza vaccine they did prove helpful in establishing new standards to be required for future ts influenza vaccine viruses, namely, genetic stability in doubly seronegative individuals and failure to induce a febrile response in such persons. Other ts mutants which contain two independent ts lesions in regions of the genome which do not code for the haemagglutinin or neuraminidase antigen are currently under study and several appear to be more stable genetically than the ts-1[E] virus. These more stable recombinants will be evaluated in volunteers shortly.

Acknowledgments

The authors wish to thank Mrs Helen Lester, Mrs Mary Mullinix, Mr Isaac Fishburne, Mr Lee Cline and Mrs Eveline Tierney for their excellent assistance. They also wish to thank Drs Gordon Douglas, Jack Gwaltney, and Neil Blacklow for their collaboration in the volunteer studies.

References

- FOY, H.M., COONEY, M.K. & McMAHAN, R. (1973) A/Hong Kong influenza immunity three years after immunization. *Journal of the American Medical Association*, **226**, 758.
- HALL, C.E., COONEY, M.K. & FOX, J.P. (1973) The Seattle Virus Watch IV. Comparative epidemiologic observations of infections with influenza A and B virus, 1965-1969, in families with young children. *American Journal of Epidemiology*, **98**, 365.
- HENNESSEY, A.V. & DAVENPORT, F.M. (1967) Vaccination of infants against influenza with polyvalent influenza hemagglutinin. *Journal of the American Medical Association*, **200**, 896.
- HENNESSEY, A.V. & DAVENPORT, F.M. (1974) Studies on vaccination of infants against influenza with influenza hemagglutinin. *Proceedings of the Society for Experimental Biology and Medicine*, **146**, 200.
- HIRST, G.K. (1973) Mechanism of influenza 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.
- MAASSAB, H.F. (1969) Biologic and immunologic characteristics of cold adapted influenza virus. *Journal of Immunology*, **102**, 728.
- MACKENZIE, J.S. (1969) Virulence of temperature-sensitive mutants of influenza virus. *British Medical Journal*, **3**, 757.
- MCCAHON, D. & SCHILD, G.C. (1972) Segregation of antigenic and biological characteristics during influenza virus recombination. *Journal of General Virology*, **15**, 73.

- MILLS, J.V., CHANOCK, R.M. & ALLING, D.W. (1969) Mutants of influenza virus. *British Medical Journal*, **4**, 690.
- MILLS, J.V. & CHANOCK, R.M. (1971) Temperature-sensitive mutants of influenza. I. Behavior in tissue culture and in experimental animals. *Journal of Infectious Diseases*, **123**, 145.
- MONTO, A.S., DAVENPORT, F.M., NAPIER, J.A. & FRANCIS, T. (1969) Effect of a school-age population upon the course of an A2/Hong Kong influenza epidemic. *Bulletin of the World Health Organization*, **41**, 537.
- MURPHY, B.R., CHALHUB, E.G., NUSINOFF, S.R. & CHANOCK, R.M. (1972) Temperature-sensitive mutants of influenza virus. II. Attenuation of ts recombinants for man. *Journal of Infectious Diseases*, **126**, 170.
- MURPHY, B.R., CHALHUB, E.G., NUSINOFF, S.R., KASEL, J. & 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. *Journal of Infectious Diseases*, **128**, 479.
- MURPHY, B.R., HODES, D.S., NUSINOFF, S.R., SPRING-STEWART, S.B., TIERNEY, E.L. & 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. *Journal of Infectious Diseases*, **130**, 144.
- MURPHY, B.R., SPRING, S.B., RICHMAN, D.D., TIERNEY, E.L., KASEL, J. & CHANOCK, R.M. (1975) Temperature-sensitive mutants of influenza virus. VII. Transfer of the ts-1[E] lesions to a wild type influenza A virus with the H₀N₁ surface antigens. *Virology*, **66**, 533.
- RICHMAN, D.D., MURPHY, B.R., SPRING, S.B., COLEMAN, M.T. & CHANOCK, R.M. (1975) Temperature-sensitive mutants of influenza virus. IX. Genetic and biological characterization of ts-1[E] lesions when transferred to 1972 (H₃N₂) influenza A virus. *Virology*, **66**, 551.
- SPRING, S.B., NUSINOFF, S.R., MILLS, J.V., RICHMAN, D.D., TIERNEY, E.L., MURPHY, B.R. & CHANOCK, R.M. (1975a) Temperature-sensitive mutants of influenza virus. VI. Transfer of ts lesions from the Asian subtype of influenza A virus (H₂N₂) to the Hong Kong subtype (H₃N₂). *Virology*, **66**, 522.
- SPRING, S.B., NUSINOFF, S.R., TIERNEY, E.L., RICHMAN, D.D., MURPHY, B.R. & CHANOCK, R.M. (1975b) Temperature-sensitive mutants of influenza virus. VIII. Genetic and biological characterization of ts mutants of influenza virus A (H₃N₂) and their assignment to complementation groups. *Virology*, **66**, 542.
- SUGIURA, A., TOBITA & KILBORNE, E.D. (1972) Isolation and preliminary characterization of temperature-sensitive mutants of influenza virus. *Journal of Virology*, **10**, 639.
- WRIGHT, P.F., SHINOZAKI, T., THOMPSON, T.M. & KARZON, D.T. (1975) Live influenza A (H₃N₂) ts-1[E] vaccine in children. *Pediatric Research*, **9**, 347.