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Occurrence of Temperature-Sensitive Influenza A Viruses in Nature

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The origin and characteristics of the first naturally occurring temperature-sensitive (*ts*) strain of influenza A virus identified in 1973, Xia-*ts*, are described. Natural *ts* strains were found to occur in the early egg passage material of all influenza A subtypes examined, but the proportion of *ts* virus varied from 8.3% for old H1N1 virus (1949 to 1957) to 82.4% for recent H3N2 virus (1979 to 1980). A number of strains were found to be composed of a mixture of *ts* and wild-type (ts^+) particles. Six natural *ts* strains with different shutoff temperatures and one ts^+ strain of the H1N1 subtype were tested in antibody-free volunteers. Strains with a shutoff temperature of 38°C or lower caused very mild symptoms, whereas those with a shutoff temperature of 39°C and the ts^+ strain were much more reactogenic. By complementation tests against a set of prototype WSN *ts* mutants with a defined genetic lesion, the *ts* lesion of two H3N2 viruses (HK/8/68 and Xia-*ts*) was located on the NP gene and that of two H1N1 viruses (Tianjin/78/77 and Beijing/1/79) was located on the M protein gene. The present study demonstrates the widespread occurrence in nature of influenza viruses of different degrees of temperature sensitivity and presumably of different degrees of virulence.

Temperature-sensitive (*ts*) mutants are generally obtained by mutagenesis from wild-type (ts^+) virus. A naturally occurring influenza A strain was first discovered in 1973 from a subclone of freshly isolated H3N2 virus, Ningxia/11/72 (15). This strain, called Xia-*ts*, had been studied extensively as the master strain for recombinant live influenza vaccine. Another *ts* strain of H3N2 virus was identified in 1976 (15). However, it was not until 1977, when we found that many fresh isolates from the epidemic caused by the recurrence of H1N1 were *ts* (4), that our attention was directed to the general nature of this phenomenon. Accordingly, a systematic study was carried out to examine the *ts* character of a number of influenza A strains of different subtypes isolated in different years. As a part of our live vaccine studies, an attempt was also made to determine the virulence of several strains of H1N1 virus in relation to their *ts* character in antibody-free human volunteers. Finally, the genetic location of the *ts* lesion of the currently circulating H1N1 and H3N2 viruses was determined by complementation-recombination tests against a set of WSN *ts* mutants with a known single-gene lesion. The results of these studies are reported in this paper.

MATERIALS AND METHODS

Viruses. Many strains of influenza A virus were screened for their *ts* character, including 12 old H1N1 strains from 1949 to 1957, 32 new H1N1 strains from 1977 to 1979, 16 H2N2 strains from 1957 to 1967, and 57 strains of H3N2 from 1968 to 1980. These strains will not be named individually. However, it is important to note (Table 1) that, of these 117 strains, 46 were within 3 egg passages from their initial isolation, 27 were within 5 passages, 30 were within 10 passages, and only 6 were over 10 passages. In fact, many of the recent H1N1 and H3N2 viruses were examined in their first or second passage. Viruses were passed routinely at 10^{-3} in the allantoic cavity of 9- to 11-day-old chicken embryos.

WSN mutants. WSN mutants were originally developed by Sugiura et al. (14) and kindly supplied by P. Palese (Mount Sinai School of Medicine, New York); they possess a *ts* lesion on a single defined gene. Their complementation groups, genetic lesions, defective proteins, and functions are given in Table 2. *ts*61S was markedly leaky; *ts*6, *ts*53, *ts*101, *ts*3, and *ts*51 were moderately leaky; and *ts*15 and *ts*56 were practically nonleaky. Stocks of these mutants were prepared in chicken embryos by selecting individual allantoic fluids with the least leakiness and the lowest reversion rate.

Examination of *ts* character. The *ts* character of the virus strains was examined routinely by parallel titration in chicken embryos at 33 and 39°C, using four eggs for each dilution. The 50% egg infective dose (EID_{50}) per 0.2 ml at each temperature was calculated. Strains with a 33°C/39°C log EID_{50} difference of <2.5 are considered ts^+, those with a difference of >3.5 are considered *ts*, and those with a difference intermediate between 2.5 and 3.5 are considered ts^*. A number of strains were also examined by parallel plaque assay in

TABLE 1. Passage histories of influenza A virus strains examined for *ts* character

Subtype	Time of isolation (yr)	No. of strains examined	No. with following egg passage history:				
			≤E3	≤E5	≤E10	>E10	Unknown
H1N1	1949–1957	12	1	3	5		3
H2N2	1957–1967	16		2	9	4	1
H3N2	1968–1978	23	6	8	6	2[a]	1
H3N2	1979–1980	34	23[b]	8	3		
H1N1	1977.6–1978.2[c]	17	8	2	6		1
H1N1	1978.6–1979.1	15	8	4	1		2

[a] These two strains were initially isolated in monkey kidney cells, with 11 subsequent egg passages.
[b] One of these strains was initially isolated in human embryonic kidney cells and passed once in eggs.
[c] Figures following the year indicate the month of virus isolation.

chicken embryo cells at 33 and 39.5°C. Strains with a 33°C/39.5°C log PFU difference of >2.0 are considered *ts*. The reason for using 39.5°C in this case is because some *ts* strains showed diffuse indistinct plaques at 39°C.

Plaque assay. Plaque assay was performed as described previously (5). Chicken embryo cell monolayers in rubber-stoppered bottles were infected with 1 ml of 10-fold dilutions of virus. Adsorption was allowed to proceed at 33°C for 1 h. Virus was discarded, and the cell monolayer was washed twice before being overlaid with 1% agarose containing 1:25,000 neutral red and 10 μg of trypsin per ml. The bottles were incubated at either 33 or 39.5°C, and plaques were counted after 3 to 4 days.

Complementation-recombination tests. Complementation-recombination tests were performed as described by Spring et al. (12). Our natural *ts* strains were surprisingly nonleaky, even at 10^{-1}. This was taken advantage of when the test was carried out with a somewhat leaky mutant of WSN as the partner. Complementation could be demonstrated by mixed infection with a fixed dilution (usually 10^{-1} or 10^{-2}) of the former against several dilutions (e.g., 10^{-3}, 10^{-4}, and 10^{-5}) of the latter. This was probably due to the participation of noninfective particles in the complementation-recombination process. The complementation index was calculated as the ratio of the PFU obtained after mixed infection to the sum of the PFU of single infections at 39.5°C, or AB/(A + B). Both viruses were also assayed at 33°C to quantitate the amount of infective virus present. A complementation index of ≥5 was taken arbitrarily as the base line for significant complementation.

Clinical trials in volunteers. Chicken embryonic allantoic fluid virus was diluted to contain approximately $10^{7.0}$ EID_{50}/0.5 ml and administered intranasally by a coarse spray. Healthy young adult volunteers 18 to 22 years of age were bled before inoculation and again 3 weeks after inoculation. For Xia-*ts* (H3N2), volunteers with a pre-inoculation homologous hemagglutination inhibition titer of <1:5 were selected. They probably possessed some antibody to previous H3N2 variants. For H1N1 virus, volunteers of the same age and hemagglutination inhibition titer probably had never been infected and were thus truly antibody-free. Clinical reactions were observed for 4 consecutive days after inoculation, and the body temperature was measured twice daily. A maximal temperature of ≤37.2°C during the observation period was considered normal. A maximal temperature of 37.3 to 37.5°C was considered a mild reaction, one of 37.6 to 38.5°C was considered a moderate reaction, and one of ≥38.6°C was considered a strong reaction. A fourfold or more rise in antibody was taken as an index of immunogenicity.

RESULTS

Characterization of a naturally occurring H3N2 *ts* strain. The first naturally occurring *ts* strain was found in a subclone of an H3N2 virus, Ningxia/11/72 (E1T2E3). This subclone, Xia-*ts*, was obtained by three successive terminal dilution passages in chicken embryos at 33°C. Another subclone, Xia-ts^+, was obtained by treatment with dimethyl sulfate and passed once in chicken embryos at 25°C, followed by two terminal dilution passages at 25°C and one terminal

TABLE 2. Single-lesion mutants of WSN (H1N1) and their complementation groups, genetic lesion, and functional defect

Complementation group	Representative mutant(s)	Genetic lesion	Defective protein[a]	Functional defect
I	*ts*6	RNA-1	P3	Synthesis of cRNA
II	*ts*53	RNA-3	P2	Synthesis of vRNA
III	*ts*15, *ts*101	RNA-2	P1	Synthesis of cRNA
IV	*ts*3	RNA-6	NA	Splits off acetylneuraminic acid
V	*ts*56	RNA-5	NP	Synthesis of vRNA
VI	*ts*61S	RNA-4	HA	Adsorption to cell
VII	*ts*51	RNA-7	M	Assembly?

[a] P1, P2, and P3, RNA polymerase; NA, neuraminidase; NP, nucleoprotein; HA, hemagglutinin; M, matrix protein.

TABLE 3. Titer difference of the Ningxia/11/79 (H3N2) wild type and its ts^+ (Xia-ts^+) and *ts* (Xia-*ts*) subclones at permissive (33°C) and nonpermissive (39°C) temperatures

Temp (°C)	Wild type (EID_{50}[a])	Xia-ts^+		Xia-*ts*	
		EID_{50}	$TCID_{50}$[b]	EID_{50}	$TCID_{50}$
33	7.75	8.00	6.00	6.68	4.75
39	8.50	7.37	5.50	≤2.83	1.25
Titer difference	−0.75	0.63	0.50	≥3.85	3.50

[a] EID_{50}, Log 10 of 50% egg infective dose in 0.2 ml.
[b] $TCID_{50}$, Log 10 of 50% tissue culture infective dose on hamster kidney cell monolayers in 0.1 ml.

dilution passage at 33°C. It was our initial intention to obtain a cold-adapted variant in this way, but to our surprise, a ts^+ subclone turned up. This curious result may be partially explained by our later finding that Xia-*ts* was more restricted than Xia-ts^+ not only at 39°C, but also at 25°C. The infective titers of Xia-*ts*, whether assayed in chicken embryos or in hamster kidney cells, were more than 3 logs lower at 39°C than at 33°C (Table 3). Xia-*ts* replicated very little at 39°C (Table 4).

For a determination of the shutoff temperature for Xia-*ts*, this virus was titrated in chicken embryos at 33, 38, and 39°C, and its EID_{50} was found to be 7.0, ≤2.5, and ≤1.5, respectively, indicating a shutoff temperature of ≤38°C.

The replication of Xia-*ts* and Xia-ts^+ in golden hamster lungs was determined by inoculating $10^{5.4}$ EID_{50} of virus per 0.05 ml intranasally and harvesting the lungs 24, 48, and 72 h later. The lungs from two animals in each group were pooled, triturated to a 10% suspension, centrifuged, and titrated in chicken embryos. Xia-*ts* replicated more slowly and to a maximal titer 2 logs lower than did Xia-ts^+ (Fig. 1).

Xia-*ts* was passed successively in chicken embryos at 33 and 37°C for six passages and found to remain *ts*, indicating that its *ts* character is genetically fairly stable.

A preparation of Xia-*ts* (E1T2E3T3E2) was examined as for the control of live vaccine. This virus was then tested in 11 low-antibody volunteers. Two mild reactions and one strong reaction were noted, and 8 of 11 volunteers responded with a fourfold or more rise in antibody. Virus was isolated from four volunteers, and the two isolates tested remained *ts*. These findings seem to demonstrate that Xia-*ts* is markedly, but not completely, attenuated for humans.

Occurrence of *ts* strains in different subtypes and during various periods. The proportions of *ts* virus among strains of different subtypes and isolated during different periods are shown in Table 5. Only 1 of 12 strains of old H1N1 virus isolated during 1949 to 1957 was found to be *ts*, whereas 13 of 17 strains (76.5%) of new H1N1 virus isolated during the first year after its recurrence (June 1977 to February 1978) were *ts*; this proportion was reduced to 6 of 15 strains (40%) isolated in the next year (June 1978 to January 1979). Among the H2N2 viruses, 6 of 16

TABLE 4. Replication of Xia-ts^+ and Xia-*ts* at permissive (33°C) and nonpermissive (39°C) temperatures[a]

Replicating temp (°C)	Xia-ts^+		Xia-*ts*	
	HA[b]	EID_{50}	HA	EID_{50}
33	224	9.00	114	7.75
39	30	7.25	1	≤2.50

[a] Ten chicken embryos were infected intra-allantoically with 1,000 EID_{50} of Xia-ts^+ or Xia-*ts* per 0.2 ml. Five of each group were incubated at 33°C, and five were incubated at 39°C. Allantoic fluids were harvested after 48 h, and their hemagglutinin (HA) titers were determined individually. Fluids from each group were pooled, and the EID_{50} of the pooled virus was determined.
[b] Geometric mean of HA titers of allantoic fluid from five eggs.

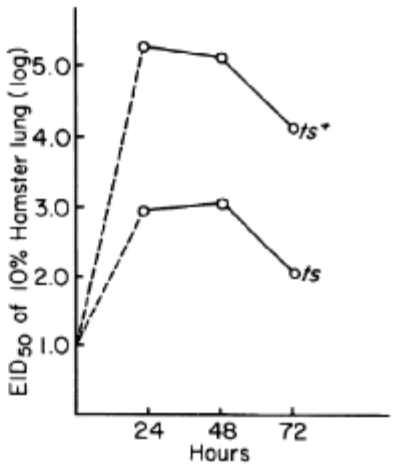

FIG. 1. Replication of Xia-ts^+ and Xia-*ts* in hamster lungs.

TABLE 5. Identification of *ts* strains in different subtypes of influenza A virus isolated during different periods

Subtype	Time of isolation (yr)	No. of strains tested	No. with following *ts* character:			
			ts	ts^-	ts^+	% *ts*
H1N1	1949–1957	12	1[a]	0	11	8.3
H2N2	1957–1967	16	6	3	7	37.5
H3N2	1968–1978	23	8	5	10	34.8
H3N2	1979–1980	34	28	3	3	82.4
H1N1	1977.6–1978.2[b]	17	13	2	2	76.5
H1N1	1978.6–1979.1	15	6	3	6	40.0

[a] Strain Kaifeng/5/56 was tested twice, was once ts^+ and once *ts*, and was later found to be a mixture of *ts* and ts^+ particles.
[b] For explanation, see Table 1, footnote *c*.

(37.5%) were *ts*, and the occurrence of the *ts* character seems to be independent of the γ-inhibitor sensitivity of the strains concerned. Among the H3N2 strains, 8 of 23 (34.8%) isolated during 1968 to 1978 were *ts*, but this proportion suddenly increased to 28 of 34 (82.4%) during 1979 to 1980.

To confirm the results of *ts* tests in chicken embryos, we performed parallel tests by plaque assay with 19 virus strains, including 8 H3N2 *ts* strains, 7 H1N1 *ts* strains, and 4 ts^+ controls. The results shown in Fig. 2 revealed complete agreement of these two methods in all cases.

Mixed composition of virus strains. The history of the isolation of Xia-*ts* and Xia-ts^+ already suggests that some virus strains may be composed of a mixture of *ts* and ts^+ particles. Further evidence of this point is shown in Table 6. In these instances, cloning by terminal dilution at 33°C yielded a *ts* virus, whereas cloning by terminal dilution at 39°C yielded a ts^+ virus. Although not studied systematically, we also have evidence indicating that different particles in a strain may vary in their shutoff temperatures. For example, of seven plaques of an H3N2 strain (Shanghai/12/81) picked and subinoculated into eggs, two plaques were shown to have a shutoff temperature of 38°C, four plaques had a shutoff temperature of 39°C, and one was ts^+.

Virulence of naturally occurring *ts* virus for humans. *ts* mutants are usually attenuated in virulence (2), and this property has been used in the development of live vaccine strains for influ-

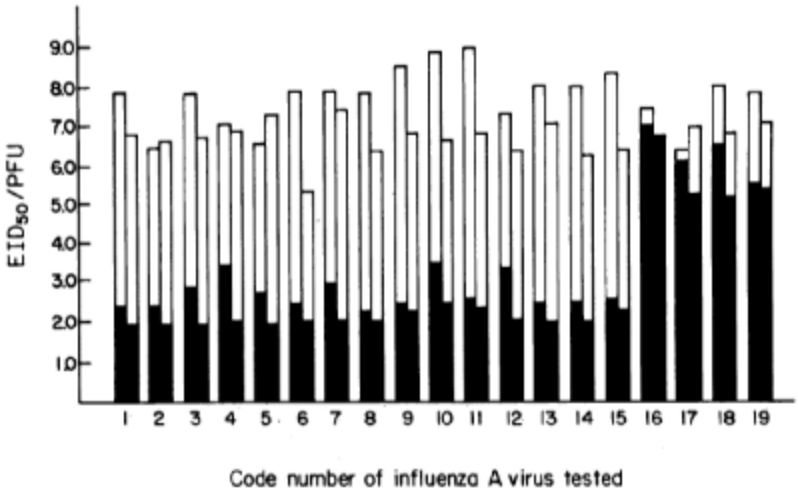

FIG. 2. Parallel test for *ts* character of influenza A strains by egg titration and by plaque assay in chicken embryo cells. The following influenza A strains were titrated in parallel: 1 to 8, H3N2; 9 to 15, H1N1; 16 to 19, ts^+ controls. 1, HK/8/68; 2, Eng/42/72; 3, Beijing/5/74; 4, Tianjin/126/79; 5, Qiqihaer/39/79; 6, Shanghai/1/80; 7, Beijing/6/80; 8, Xia-*ts*; 9, Shanxi/51/77; 10, Tianjin/78/77; 11, Haerbin/9/77; 12, Shanghai/13/78; 13, Beijing/1/79; 14, Shanghai/17/79; 15, Shanghai/19/79; 16, WSN (H0N1); 17, Fukien/8/58 (H2N2); 18, Hubei/1/72 (H3N2); 19, Victoria/2/79 (H1N1). For each virus strain, column 1 represents the EID_{50} per 0.2 ml, column 2 represents PFU per milliliter, the blank represents the titer at 33°C, and the shaded area represents the titer at 39°C (for egg titration) or 39.5°C (for plaque assay).

TABLE 6. Isolation of *ts* and *ts*$^+$ subclones from the same strains of influenza A virus

Subtype	Strain	Passage history[a]	EID_{50}		Temp sensitivity
			33°C	39°C	
H2N2	Beijing/22/57	E6	≥8.5	≤3.8	*ts*
		E6T1	8.8	3.3	*ts*
		E6(39)T1	7.8	5.5	*ts*$^+$
H3N2	Beijing/26/79	E2	7.3	4.0	*ts*$^\pm$
		E2T1E1	8.0	2.5	*ts*
		E2(39)T1E1	7.3	5.3	*ts*$^+$
H1N1	Beijing/11/77	E3	8.5	3.7	*ts*
		E3	8.0	6.0	*ts*$^+$
		E3T1E2	8.0	3.0	*ts*
		E3(39)T1	6.7	≥6.5	*ts*$^+$
H1N1	Jinan/1/78	E3	6.8	5.5	*ts*$^+$
		E3T2	6.5	≤3.5	*ts*$^\pm$
		E3T2E1	8.7	≤2.5	*ts*
		E3(39)T1	7.0	6.7	*ts*$^+$

[a] E, Number of passages in eggs at 33°C; T, number of passages at a terminal dilution at 33°C; (39)T, number of passages at a terminal dilution at 39°C.

enza and respiratory syncytial viruses (1, 7, 8). It is of obvious interest to determine whether the *ts* character of the naturally occurring *ts* influenza virus is in any sense related to its virulence and whether a suitable live vaccine virus can be selected from natural *ts* strains. For this purpose, we chose seven H1N1 strains isolated in 1977 and tested them for temperature sensitivity after one terminal dilution. Four had a shutoff temperature of ≤38°C, two had a shutoff temperature of 39°C, and one was *ts*$^+$. These viruses were further passed twice in chicken embryos, and their *ts* character and shutoff temperatures remained unchanged. They were administered to groups of antibody-free volunteers aged 18 to 22 years. The first four strains with a shutoff temperature of ≤38°C generally gave very little reaction but were variable in eliciting an antibody response (Table 7). The two strains with a shutoff temperature of 39°C and the single *ts*$^+$ strain, on the other hand, were quite reactogenic and, in general, gave rise to better antibody responses. It seems unlikely that the minor difference in chicken embryo passage history before terminal dilution could have any serious influence on the behavior of these viruses in humans. Thus, these results strongly suggest that the *ts* character runs in parallel with attenuated virulence and that strains with a shutoff temperature of ≤38°C are generally non-reactogenic for humans.

Location of *ts* lesion by the complementation-recombination test. Two strains each of H3N2 and H1N1 viruses were tested by the complementation-recombination test with seven single-gene *ts* mutants of WSN virus. Both H3N2 strains, HK/8/68 and Xia-*ts*, failed to complement with *ts*56, which was interpreted to mean

TABLE 7. Reactogenicity and immunogenicity of natural *ts* strains of H1N1 virus in antibody-free human volunteers

Strain	Passage history[a]	Shutoff temp (°C)	No. of volunteers inoculated	No. with following temp reaction[b]:			No. with fourfold antibody rise
				Mild	Moderate	Strong	
Tianjin/78/77	E5T1E2	38	13	1	0	0	7
Dandong/5/77	E5T1E2	38	11	0	1	0	8
Guizhou/16/77	E3T1E2	38	13	0	0	0	7
Haerbin/9/77	E3T1E2	38	11	0	1	1	3
Beijing/11/77	E3T1E2	39	11	2	3	2	8
Beijing/6/77	E3T1E2	39	18	4	3	2	13
Beijing/4/77	E6T1E2	*ts*$^+$	20	2	3	4	13

[a] For explanation, see Table 6, footnote *a*.

[b] Body temperature was measured twice a day for 4 successive days. Reactions: mild, maximal temperature of 37.3 to 37.5°C; moderate, maximal temperature of 37.6 to 38.5°C; strong, maximal temperature of ≥38.6°C.

that they have a *ts* lesion on the NP gene, and both H1N1 strains, Tianjin/78/77 and Beijing/1/79, failed to complement with *ts*51, which was interpreted to mean that they have a *ts* lesion on the M protein gene (Table 8). Complementation between Xia-*ts* and *ts*61S was not carried out because of the serious leakiness of the latter at the time of the experiment, but a *ts* lesion on the HA gene for Xia-*ts* was ruled out by the fact that a number of recombinants with H3 replaced by wild-type H2 or H1 remained *ts*.

DISCUSSION

Simpson and Hirst (11) reported that the rate of occurrence of a spontaneous *ts* mutant without mutagenesis in WSN is about 0.5%. This virus had undergone many passages in ferrets, mouse lung, mouse brain, and chicken embryos; consequently, it is very difficult to interpret the significance of these spontaneous *ts* mutants. Other authors (6, 13) failed to detect such spontaneous mutants. After the isolation by cloning of two *ts* strains among newly isolated H3N2 viruses in 1973 and 1976, we found 16 of 23 fresh isolates of H1N1 recurring in 1977 to be *ts* (4). The occurrence of such a large number of *ts* strains among fresh isolates is a new finding which has been confirmed by Oxford et al. (9). The existence of *ts* strains has now been extended to all influenza A subtypes examined. The *ts* character of many strains determined by titration in chicken embryos has been fully confirmed by plaque assay. Since most strains examined had fewer than 10 egg passages and some were in passage 1 or 2, it seems likely that the *ts* virus actually exists in nature. It also seems likely that, under natural conditions, many cases exist as mixtures of *ts* and ts^+ particles and possibly also as particles of different shutoff temperatures.

Recent studies of *ts* live influenza vaccines by Murphy et al. (7, 8) and by us (15) have clearly demonstrated that *ts* mutants of influenza A virus are generally attenuated and that the shutoff temperature can be taken as an index of the degree of attenuation. Our clinical trial with a ts^+ strain and a number of *ts* strains of different shutoff temperatures confirmed that this is also the case with naturally occurring *ts* strains. This presumably means that influenza viruses of different degrees of virulence exist in nature. Such a postulate may have wide implications in the interpretation of the clinical and epidemiological behavior of influenza virus. For example, we have presented epidemiological and serological evidence that, in 1977, inapparent infection with H1N1 virus was unusually prevalent among completely susceptible schoolchildren, and a *ts* strain of H1N1 virus was actually isolated from a symptomless case (10).

It is more difficult to judge the significance of the apparent fluctuations in the proportions of *ts* virus among different subtypes and during different periods. The number of strains of old H1N1, H2N2, and previous H3N2 (1968 to 1978) examined is probably too small in view of the long time span of their prevalence. However, the reduction in the proportion of *ts* virus of new H1N1 during year 2 of its circulation and the

TABLE 8. Complementation-recombination tests of naturally occurring *ts* strains of the H1N1 and H3N2 subtypes with single-lesion *ts* mutants of WSN

Strain under study	Expt no.	Complementation index[a]							
		*ts*6	*ts*53	*ts*101	*ts*15	*ts*3	*ts*56	*ts*61S	*ts*51
Tianjin/78/77 (H1N1)	1	≥300	≥300	57		14	116	84	0
	2	82	49	22		6	24	96	0
	3	38	96	10		39			0
Beijing/1/79 (H1N1)	1	27	324	14		2	58	66	0
	2	7	31	0		2	10	8	0
	3			10		8		189	0
	4			0		49			0
	5					9			0
HK/8/68 (H3N2)	1	41	19	48		18	0	4	67
	2	58	≥150	34		29	0	121	15
	3	93	25	143		51	0		37
Xia-*ts* (H3N2)	1	>7	13	>210	19,300	13	0.6		102
	2	7.8	6.1		24,800	68	0		2,750
	3	2.4	>16		>1,000		0		>70
	4		29				0		232

[a] Complementation indices between a pair of viruses at 39.5°C. 0, No plaque demonstrable at 39.5°C after mixed infection at the highest concentrations tested, usually at 10^{-1} or 10^{-2}.

marked increase of *ts* virus among H3N2 viruses isolated in 1979 to 1980 probably reflect true patterns. The former may be explained by the replacement of less virulent *ts* virus by the more virulent ts^+ virus through natural selection. At present, there is no satisfactory explanation for the latter. Preliminary experiments have revealed that the *ts* lesion of the 1970 to 1980 H3N2 isolates, in contrast to previous H3N2, is not on the NP gene. The possibility of recombination between cocirculating H3N2 and H1N1 needs further investigation.

By the complementation-recombination test, the *ts* lesion of two H3N2 strains was located on the NP gene, whereas that of two new H1N1 strains was located on the M protein gene. It is thus reasonable to speculate that each subtype may have a characteristically different location of its *ts* lesions. Further studies with more strains and a different methodology may be necessary to reach a definite conclusion. Because no influenza A virus with a *ts* lesion on the nonstructural protein (NS) gene is available, it is not possible to know whether an additional *ts* lesion exists on the NS gene.

Flamand (3) reported the identification of a number of spontaneous *ts* strains of vesicular stomatitis virus. From her report, however, it is clear that these *ts* mutants represent separate clones of the same strain, and no study has been carried out to relate the *ts* character of spontaneous mutants to virulence or to genetic lesions.

To our knowledge, the present paper represents the first report of the identification of a large number of *ts* strains among fresh isolates of a human virus. Further studies on the natural occurrence of *ts* strains in other human viruses appear to be warranted.

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