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Temperature-Sensitive Mutants of Vesicular Stomatitis Virus Are Conditionally Defective Particles That Interfere with and Are Rescued by Wild-Type Virus

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Temperature-sensitive (ts) mutants of vesicular stomatitis virus belonging to complementation groups I, II, and IV inhibited the replication of wild-type vesicular stomatitis virus when mixed infections were carried out in BHK-21 cells at 32, 37, and 39.5 C. The group IV mutant (ts G 41) was most effective in this regard; wild-type virus yields were inhibited almost 1,000-fold in mixed infections with this mutant at 32 C. In the case of group I and II mutants, inhibition of wild-type virus replication at 37 and 39.5 C was accompanied by an enhancement (up to 15,000-fold) of the yields of the coinfecting ts mutant. The yields of the group IV mutant (ts G 41) were not enhanced by mixed infections with wild-type virus at any temperature, although this mutant inhibited wild-type virus replication at all temperatures. The dominance of the replication of ts mutants at 37 C provides a rationale for the selection and maintenance of ts virus in persistently infected cells.

In 1973 Preble and Youngner reported that specific interference occurred between wild-type Newcastle disease virus (NDV_0)and clones of temperature-sensitive (ts) mutants (NDV_{pi}) spontaneously selected during persistent infection initiated in mouse L cells with NDV_0 (5). In assays of mixed progeny done in chicken embryo (CE) cell monolayer cultures, NDV_0 (>2 mm) and NDV_{pi} (<1.5 mm) plaques could be readily distinguished and tallied separately. In mixed NDV_0 and NDV_{pi} infections at 37 C, the yield of NDV_0 was reduced by 94%. The yield of NDV_{pi} was less affected by the mixed infection with NDV_0. Further investigation of this phenomenon revealed that the interference in doubly infected cells was prior to or at the level of RNA synthesis. NDV_{pi} clones consistently interfered with the replication of NDV_0 in CE cells at both the permissive (37 C) and the nonpermissive temperatures (43 C). The interference was more dramatic at 43 C.

Prior to this report, Durand (3) and Bratt and Rubin (1, 2) had speculated that interference by an avirulent NDV with multiplication of a virulent strain of virus was due to competition in the mixed infection for replicative sites or substrates. Replication of the interfering avirulent virus was necessary for development of maximum interference.

In 1974, Stollar and his colleagues (8) reported that a cloned, small-plaque, ts mutant of Sindbis virus derived from persistently infected *Aedes aegypti* cell cultures interfered with the replication of the wild-type (large plaque) virus in BHK-21 cells. This interference occurred at the nonpermissive (39.5 C) as well as the permissive (34 C) temperature but did not depress net viral RNA synthesis in BHK-21 cells coinfected with wild-type Sindbis virus and the ts mutant.

We have recently demonstrated the important role played by ts virus in the establishment and maintenance of persistent infection of L cells with vesicular stomatitis virus (VSV) (10). In an attempt to explain how ts mutants are able to establish and maintain persistent infections and why revertants do not replace the ts population, a hypothesis was offered that ts mutants interfere with wild-type virus replication and are "rescued" by the wild-type virus. This paper documents the "dominance" of ts virus over wild-type virus replication in BHK-21 cells coinfected with wild-type VSV and ts mutants representing different complementation groups of this virus.

MATERIALS AND METHODS

Cells. Primary CE cells and a line (BHK-21) of hamster kidney cells were propagated in Eagle minimal essential medium plus 4% calf serum.

Viruses. A cloned population (WT $_0$3) of a large-plaque mutant of VSV_{IND} (9) was grown in BHK-21 cells and assayed in CE cells. All virus pools were produced using inocula corresponding to less than 0.01 PFU/cell to avoid production of significant numbers of defective interfering particles. ts mutants of VSV_{IND} representing RNA^- complementa-

tion groups I, II, and IV were obtained from R. R. Wagner. Mutant ts pi 26, another group I mutant, was isolated from a persistent infection of L cells with VSV, as described previously (10). The clones used and their characteristics are summarized in Table 1.

Mixed infection of BHK-21 cells with wild-type VSV and ts mutants. BHK-21 cell monolayers were grown at 37 C in plastic trays (Disposo-trays, Linbro Chemical Co.) containing 24 wells of 16-mm diameter; from 5×10^5 to 10^6 cells were present in each well when the monolayers were confluent. Duplicate wells were infected with 0.2 ml of $WT_0 3$ (multiplicity of infection = 0.1) or a ts mutant (multiplicity of infection = 1.0) alone or with 0.2 ml of mixtures containing virus concentrations, to give the same multiplicity of infection as for single infections. Virus samples were diluted in growth medium plus 10 μg of DEAE-dextran per ml. After an adsorption period of 2 h at 37 C, the inoculum was removed. The monolayers were washed twice with warm medium, 1 ml of warm medium was added, and the trays were transferred to the appropriate temperature in a humidified incubator gassed with CO_2. Replicate trays with single- and mixed-infection wells were incubated at 32, 37, and 39.5 C for 20 h, at which time the cell destruction at 32 C was complete. The fluid in paired wells was pooled, and the infectivity was determined by plaque assay in CE cells at 32 and 39.5 C. At terminal dilutions, plaques were scored according to size, as indicated in Table 1; validation of this method for differentiating wild-type and ts mutant plaques will be described below.

Screening of VSV clones for temperature sensitivity. CE cell monolayers were grown at 37 C in plastic trays containing 24 wells of 16-mm diameter. Each well was seeded with 1.5 ml of a standard dilution of primary CE cells; from 8×10^5 to 10^6 cells were present in each well when the monolayers were confluent. The cells in each well were infected with 0.1 ml of undiluted virus suspension from isolated plaques in 1.0 ml of medium. Replicate welled trays were incubated at 32 or 39.5 C in humidified incubators gassed with CO_2. The infected monolayers were examined under a microscope for cytopathology, which correlates with virus replication. When cytopathic effect was complete in wells incubated at 32 C (usually 48 h after infection), fluids were harvested from wells at both temperatures and assayed by the plaque technique at 32 C.

RESULTS

Mixed infection of BHK-21 cells with wild-type virus and ts G 11. BHK-21 cell monolayers in plastic welled trays were infected with a wild-type virus cloned population ($WT_0 3$) and a group I mutant (ts G 11) as described above. The analysis of progeny viruses produced by single and mixed infections with the two viruses is shown in Table 2. It is clear that at all three temperatures the replication of the wild-type virus was significantly reduced by coinfection with ts G 11. This inhibition varied from 25-fold at 32 C to 40-fold at the two higher temperatures of incubation. The ability of ts G 11 to inhibit wild-type virus replication was not affected by the reduced ability of the ts mutant to replicate at 37 and 39.5 C. This can be noted in the column showing yields from single infections with ts G 11; the 37/32 C yield ratio of the ts mutant alone was 5.5×10^{-3} and the 39.5/32 C yield ratio was 2.8×10^{-6}. Unexpectedly, at 37 and 39.5 C the progeny of mixed infections contained 44- and 7,100-fold higher concentrations of small-plaque ts virus than did the progeny of single infections with the ts virus. At 32 C, the number of ts mutant progeny was not different in single or mixed infections.

Validation of the plaque size selection method for scoring progeny virus. The progeny that resulted from the $WT_0 3 \times$ ts G 11 mixed infection at 32 C (described in Table 2) were plated in CE cell monolayers at 32 C. Five large plaques (2.5 to 3.0 mm) and five small plaques (<1 to 1.0 mm) were isolated from terminal dilution plates and tested for temperature sensitivity by the "welled tray" method described in Materials and Methods. Table 3 shows that all five large clones produced complete cell destruction at both 32 and 39.5 C, and the 39.5/32 C yield ratios tested for two of these clones were 0.28 and 0.27. In contrast, all five small clones failed to produce any cytopathic effect at 39.5 C, and the 39.5/32 C yield ratios tested for three of these clones ranged from $<4.1 \times 10^{-6}$ to $<7.6 \times 10^{-7}$ (Table 3). These results demonstrate the accuracy of the plaque size selection method for differentiating large and small plaques as $WT_0 3$ or ts G 11 progeny.

In addition to the clonal testing procedures described, confirmation of the plaque size scoring method for differentiating wild-type and ts mutant populations was provided by testing the efficiency of plating of the mixed progeny at 32 and 39.5 C (Table 4). When assayed at 39.5 C, only large plaques were obtained; no small plaques were seen on terminal dilution plates. The absence of small plaques at 39.5 C and the consistency of the 39.5/32 C efficiency of plating ratios with those of $WT_0 3$ virus further established the validity of the methods used for distinguishing $WT_0 3$ from ts G 11 progeny.

Mixed infection of BHK-21 cells with $WT_0 3$ and ts pi 26 or ts O 52. Mixed infections were carried out with $WT_0 3$ virus and ts pi 26, a group I mutant isolated from a persistently infected line of mouse L cells (10). The characteristics of ts pi 26 are listed in Table 1. At all three temperatures used, there was a five- to eightfold decrease in the number of wild-type progeny viruses produced in mixed infections (Table 5). However, a significant increase in the number of ts pi 26 clones occurred at 37 C

TABLE 1. *Characteristics of wild-type virus clone (WT_03) and ts mutants used as inocula*

Virus	Classification	Plaquing efficiency in CE cells		Yield efficiency in BHK-21 cells (39.5/32 C ratio)
		Plaque size[a] at 32 C (mm)	39.5/32 C ratio	
WT_03	Wild-type	2.5-3.0	0.74	0.31
ts G 11	Group I	<1.0-1.5	$<3.0 \times 10^{-7}$	2.9×10^{-6}
ts pi 26	Group I	<1.0-1.5	$<5.9 \times 10^{-7}$	1.3×10^{-5}
ts 0 52	Group II	1.0-1.5	2.4×10^{-6}	2.7×10^{-4}
ts G 41	Group IV	<1.0	$<6.7 \times 10^{-5}$	2.3×10^{-4}

[a] Three days at 32 C.

TABLE 2. *Analysis of progeny viruses produced by mixed infection of BHK-21 cells with WT_03 and ts G 11 at 32, 37, and 39.5 C*

Temp (C) of replication in BHK-21 cells	Plaque size[a] at 32 C (mm)	PFU/ml[a]			Ratio: mixed infection/single infection
		Single infection		Mixed infection[b]	
		WT_03[c]	ts G 11[d]	(WT_03 × ts G 11)	
32	2.5-3.0	7.6×10^8	0[e]	3.0×10^7	0.03
	1.0-1.5	0	4.9×10^8	4.7×10^8	0.95
37	2.5-3.0	8.2×10^8	0	2.0×10^7	0.02
	1.0-1.5	0	2.7×10^6	1.2×10^8	44.4
39.5	2.5-3.0	2.4×10^8	0	6.0×10^6	0.02
	1.0-1.5	0	1.4×10^3	1.0×10^7	7.1×10^3

[a] In CE cell monolayers (32 C, 3 days).
[b] Multiplicity of infection (MOI) as in footnotes *c* and *d*.
[c] MOI = 0.1.
[d] MOI = 1.0.
[e] 0, Not detected in terminal dilution plates.

(sevenfold). This rescue phenomenon in mixed infection was dramatic at 39.5 C; the yield of ts pi 26 virus was increased 15,000-fold over the yield at 39.5 C from single infection with this mutant.

It should be noted at this point that a validation of the plaque size scoring method was carried out, as described in Tables 3 and 4, using the progeny of the WT $_03$ × ts pi 26 mixed infection at 39.5 C. All five large plaques selected from terminal dilution plates proved to be wild-type viruses, and all five small plaques were ts mutants. Efficiency of plating determinations at 32 and 39.5 C, as described in Table 4 for the WT $_03$ × ts G 11 mixed infection, also indicated the validity of the plaque size differentiation criteria used to analyze the date.

When the progeny of WT $_03$ × ts O 52 mixed infection at 32, 37, and 39.5 C were evaluated as has been described, data essentially similar to those reported for WT $_03$ × ts G 11 and WT $_03$ × ts pi 26 mixed infections were obtained. Of particular note, analysis of the progeny of WT $_03$ × ts O 52 mixed infections at 39.5 C revealed that the wild-type progeny were reduced by fivefold, whereas there was a 691-fold increase in ts O 52 clones. Again, validation tests as described in Tables 3 and 4 for other mixed infections showed the accuracy of the scoring methods used.

Mixed infection of BHK-21 cells with WT $_03$ and ts G 41. A different pattern of progeny production was obtained when mixed infections at 32, 37, and 39.5 C were done using wild-type virus and a group IV mutant. Most striking was the ability of ts G 41 to interfere with the replication of wild-type virus at all the temperatures tested (Table 6). For example, at 32 C, ts G 41 inhibited WT $_03$ replication by almost 1,000-fold; at 37 and 39.5 C, the inhibition of the wild type in the mixed infection was even more dramatic. In contrast to what was seen with the other mutants tested, the replication of ts G 41 was not significantly enhanced by coinfection with wild-type virus at any temperature. Validation experiments, as described above, were also carried out with the progeny of WT $_03$ × ts G 41 mixed infections, and the data again showed the consistency of the analysis methods used.

DISCUSSION

The observations with VSV ts mutants reported in this paper confirm and extend previous findings that ts mutants of NDV (5) and Sindbis virus (8) are capable of interfering with

TABLE 3. *Validation of plaque size selection method for scoring progeny virus: mixed infection of BHK-21 cells with $WT_0$3 and ts G 11 at 32 C*

Plaque size[a] at 32 C (mm)	Clone no.	Replication in CE cells				Yield ratio 39.5/32 C
		CPE[b] at 48 h		Assay of culture fluid (PFU/ml)[c]		
		32 C	39.5 C	32 C	39.5 C	
2.5-3.0	1	++++	++++	1.3×10^8	3.7×10^7	0.28
	2	++++	++++			
	3	++++	++++			0.27
	4	++++	++++	8.7×10^7	2.4×10^7	
	5	++++	++++			
<1-1.0	6	+++	0	2.4×10^6	$<10^1$	$<4.1 \times 10^{-6}$
	7	++++	0	1.3×10^7	$<10^1$	$<7.6 \times 10^{-7}$
	8	++++	0			
	9	++++	0			
	10	++	0	6.9×10^6	$<10^1$	$<1.4 \times 10^{-6}$

[a] In CE cell monolayers (32 C, 3 days).

[b] CPE, cytopathic effects: ++++, complete cell destruction; +++, 75% cell destruction; ++, 50% cell destruction; 0, no CPE.

TABLE 4. *Validation of plaque size selection method for scoring progeny virus by determining efficiency of plating of mixed progeny by assay at 32 and 39.5 C in CE cells*

Progeny produced at:	Mixed progeny of $WT_0$3 × ts G 11 in BHK-21 cells, assayed in CE cells at:				
	32 C		39.5 C		39.5/32 C ratio
	Plaque size (mm)	PFU/ml	Plaque size (mm)	PFU/ml	
32 C	2.5-3.0	3.0×10^7	1.5-2.0	5.9×10^6	0.19
	1.0-1.5	4.7×10^8	<1.0-1.5	10^4[a]	2.1×10^{-5}
37 C	2.5-3.0	2.0×10^7	2.0-2.5	8.3×10^6	0.41
	1.0-1.5	1.2×10^8	<1.0-1.5	10^4	8.3×10^{-5}
39.5 C	2.5-3.0	6.0×10^6	2.0-2.5	1.7×10^6	0.28
	1.0-1.5	1.0×10^7	<1.0-1.5	10^4	1.0×10^{-3}

[a] Small plaques not detected on terminal dilution plates.

the replication of homologous wild-type viruses at permissive and nonpermissive temperatures. Mutants belonging to VSV complementation groups I, II, and IV inhibited to varying degrees the replication of a clone of wild-type virus ($WT_0$3) when mixed infections were carried out in BHK-21 cells at 32, 37, and 39.5 C. The group IV mutant (ts G 41) was most effective in this regard; wild-type virus yields were inhibited almost 1,000-fold in mixed infections with this mutant at 32 C. Inhibition of wild-type virus replication was obtained in mixed infections at 37 and 39.5 C with all the ts mutants tested. In the case of the group I and II mutants, this inhibition of wild-type virus replication at 39.5 C was accompanied by a marked enhancement of the yields of the coinfecting ts mutant. This enhancement of ts mutant replication at the "nonpermissive" temperature (39.5 C) reached levels of 7,100-fold in the case of ts G 11 (group I), 15,000-fold with ts pi 26 (a group I mutant isolated from persistently infected L cells), and 700-fold with ts O 52 (group II). The group IV mutant (ts G 41) inhibited wild-type replication at all the temperatures tested. However, this mutant was different from the others in one important respect: the replication of ts G 41 was not enhanced by mixed infection with the wild-type virus at any temperature.

The biochemical basis of the "dominance" of the ts mutant replication over that of the wild type is the subject of our continuing interest. The data in this report deal only with the effects of members of complementation groups made up of mutants with RNA^- phenotypes. In addition to testing other members of these groups, especially of group IV, our experiments are being extended to include viruses representing complementation groups III and V, which consist of mutants with RNA^+ phenotypes. In this regard, it should be noted that Stollar and his colleagues reported that it was an RNA^+ ts mutant of Sindbis virus that was capable of interfering with the replication of wild-type virus at the nonpermissive temperature as well as at the permissive temperature (8).

These observations concerning the dominance of the replication of ts virus over that of the wild type at 37 C must be considered a rationale for the spontaneous selection and maintenance of ts mutants in persistently infected cell lines (6, 7, 10, 11). The ability of the ts mutants to interfere with the replication of

TABLE 5. *Analysis of progeny viruses produced by mixed infection of BHK-21 cells with $WT_0$3 and ts pi 26 at 32, 37, and 39.5 C*

Temp (C) of replication in BHK-21 cells	Plaque size[a] at 32 C (mm)	PFU/ml[a]			Ratio: mixed infection/single infection
		Single infection		Mixed infection[b]	
		$WT_0$3[c]	ts pi 26[d]	($WT_0$3 × ts pi 26)	
32	2.5–3.0	7.6×10^8	0[e]	1.0×10^8	0.13
	<1.0–1.5	0	3.1×10^8	4.3×10^8	1.38
37	2.5–3.0	8.2×10^8	0	1.9×10^8	0.23
	<1.0–1.5	0	3.2×10^7	2.1×10^8	6.56
39.5	2.5–3.0	2.4×10^8	0	5.0×10^7	0.20
	<1.0–1.5	0	4.0×10^3	6.3×10^7	1.5×10^4

[a] In CE cell monolayers (32 C, 3 days).
[b] Multiplicity of infection (MOI) as in footnotes *c* and *d*.
[c] MOI = 0.1
[d] MOI = 1.0.
[e] 0, Not detected on terminal dilution plates.

TABLE 6. *Analysis of progeny viruses produced by mixed infection of BHK-21 cells with $WT_0$3 and ts G 41 at 32, 37, and 39.5 C*

Temp (C) of replication in BHK-21 cells	Plaque size[a] at 32 C (mm)	PFU/ml[a]			Ratio: mixed infection/single infection
		Single infection		Mixed infection[b]	
		$WT_0$3[c]	ts G 41[d]	($WT_0$3 × ts G 41)	
32	2.5–3.0	7.6×10^8	0[e]	1.0×10^6	1.3×10^{-3}
	<1.0	0	1.6×10^8	1.7×10^8	1.06
37	2.5–3.0	8.2×10^8	0	2.3×10^4	2.8×10^{-5}
	<1.0	0	4.0×10^5	4.0×10^5	1.00
39.5	2.5–3.0	2.4×10^8	0	9.0×10^4	3.7×10^{-4}
	<1.0	0	3.7×10^4	8.8×10^4	2.37

[a] In CE cell monolayers (32 C, 3 days).
[b] Multiplicity of infection (MOI) as in footnotes *c* and *d*.
[c] MOI = 0.1.
[d] MOI = 1.0.
[e] 0, Not detected on terminal dilution plates.

wild-type virus at 37 C would provide an answer to the question, "Why don't revertants replace the ts population at 37 C, a temperature not optimum for the ts mutants?" Since the ts mutants present in a persistent infection can inhibit the replication of wild-type virus, revertants would be prevented from providing a significant portion of the virus population. In this way, a ts virus population selected during a persistent infection could maintain its mutant character. In addition, it has been noted that strong homologous interference to challenge with wild-type virus is established in persistently infected L cells carrying ts VSV mutants (10).

The mechanism by which the ts mutants are able to establish themselves in persistent infections is still not clear. The role of defective interfering particles of VSV in the initiation of persistent infections with wild-type virus is well documented (4, 10). The ability of defective interfering particles to modulate the replication of the infective B particles may be crucial at the outset in order to prevent total cell destruction. Once this danger has passed, there may be a rapid selection of ts mutants by some intervening mechanism that is not evident. The dramatic rapidity of the selection of ts mutants during the early phases of the establishment of VSV persistence in mouse L cells has been reported in detail (10). It should be noted here that persistent infection of L cells at 37 C has been initiated in the absence of significant numbers of defective interfering particles by using low input multiplicities of a plaque-purified RNA^- group I mutant (10). In this in-

stance, conditions from the outset favored the continued maintenance of the ts mutant in the persistently infected cells. First, less than optimum replication of the ts mutant at 37 C did not result in the destruction of the infected cells. Second, the dominance of the ts mutant replication over that of the wild-type virus provided conditions for exclusion of revertant viruses. Continued efforts are being made to provide more detailed insight into the mechanisms involved in these phenomena.

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