JOURNAL OF VIROLOGY, July 1976, p. 90-101

Vol. 19, No. 1
Printed in U.S.A.

Role of Temperature-Sensitive Mutants in Persistent Infections Initiated with Vesicular Stomatitis Virus

JULIUS S. YOUNGNER,* EDWARD J. DUBOVI,[1] DIANE O. QUAGLIANA, MARION KELLY, AND OLIVIA T. PREBLE

Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received for publication 27 February 1976

Noncytocidal persistent infections at 37 C of mouse L cells (L_{VSV}) with infective B particles of vesicular stomatitis virus (VSV) could be established only in the presence of large numbers of defective interfering (DI) particles. Under these conditions, there was a rapid spontaneous selection of temperature-sensitive (ts) virus. At 10 days there was an increase to 17.8% in the frequency of ts clones in the virus population; by 17 days this frequency had reached 85.2%, and by 63 days 100% of the clones isolated were ts at 39.5 C, the nonpermissive temperature used. All 34 of the clones isolated from the 84-day fluid had an RNA^- phenotype, and 8 clones that were tested all belonged to VSV complementation group I. When tested by an interference assay, L_{VSV} fluids did not contain significant numbers of DI particles (<1 DI/PFU). Furthermore, persistent infection of L cells at 37 C could be initiated under conditions in which few, if any, DI particles were present by using low input multiplicities (10^{-4} and 10^{-5}) of a clonal isolate of an RNA^- group I mutant obtained from L_{VSV} cells. On the basis of these and other results, a mechanism is proposed to explain the role of ts mutants in both the establishment and maintenance of the persistently infected state.

Several mechanisms have been suggested that may be involved in the establishment and maintenance of persistent infection by normally virulent viruses. Huang and Baltimore (9) speculated that defective interfering (DI) particles may be involved in regulation of virus production in persistent infections. In support of their theory, Holland and Villarreal (6) have provided evidence that persistent noncytocidal infections with vesicular stomatitis virus (VSV) may be mediated by DI particles of this virus.

Another mechanism involving temperature-sensitive (ts) mutants was suggested by Preble and Youngner (15). Their studies with Newcastle disease virus (12-14, 23) showed that selection of ts mutants was involved in establishment and maintenance of persistent infections in several types of cell cultures. In addition, a survey of the literature revealed many other examples of selection of ts mutants by persistent infections, both in cell cultures and in animals (15).

More recently, several groups of workers have proposed that ordinarily cytolytic riboviruses can form infectious proviral DNA intermediates, which persist in actively dividing mammalian cells independently of detectable viral gene expression (18, 25, 26). This is considered a mechanism that would permit riboviruses to persist in eukaryotic cells for extended periods.

This report deals with the establishment of persistent infections of mouse L cells with VSV. The investigations considered the role of DI particles in establishment and maintenance of the carrier state, the selection and characteristics of ts mutants in the persistent infection, and the altered properties of the persistently infected cells.

MATERIALS AND METHODS

Cells. Primary chicken embryo (CE) cells, mouse L cells (clone 929), and a line (BHK-21) of hamster kidney cells were propagated in Eagle minimal essential medium plus 4% calf serum.

Viruses. The large-plaque mutant of VSV_{IND} (L_1VSV) described by Wertz and Youngner (22) was grown in BHK-21 cells and assayed in CE cells. Monolayers in 32-ounce (ca. 950-ml) culture bottles were infected with less than 0.01 PFU/cell to avoid production of DI particles. This wild-type virus stock is referred to in the text as VSV_0. Analysis of [^{3}H]uridine-labeled viral particles by sucrose gradients failed to detect DI particles in lysates produced under these conditions. ts mutants of VSV_{IND}

[1] Present address: Department of Microbiology, School of Medicine, University of Virginia, Charlottesville, Va. 22901.

representing known complementation groups were obtained from R. R. Wagner. Pseudorabies virus (PSR) was grown in RK-13 cells and assayed in CE cells as described previously (24).

VSV-specific RNA synthesis. Cumulative VSV-specific RNA synthesis in CE cells at 32 and 39.5 C was determined as described previously for Newcastle disease virus (13).

Screening of VSV clones for temperature sensitivity. CE cell monolayers were grown in plastic trays (Disposo-trays, Linbro Chemical Co.) 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. CE cells in control wells were mock infected or infected with 0.1 ml of a suspension of a clone of VSV_0, the wild type. 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 (CPE), which correlates with virus replication. Cultures with extensive CPE always showed virus production comparable to the wild-type virus, whereas cultures with little or no CPE produced markedly reduced amounts of virus progeny. When CPE 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. Virus clones with replication efficiencies of 10^{-2} or less at 39.5 C compared to 32 C were considered ts mutants. Where indicated, working stocks of ts mutants were prepared in CE cell monolayers in 2-ounce (ca. 60-ml) bottles. Repeated testing confirmed that the CPE screening method was extremely reliable. In cases where the CPE findings were ambiguous, yield assays of well fluids were used to classify the ts phenotype of the particular clone involved.

Production of DI particles of VSV. DI particles were produced by three serial undiluted passages of VSV_0 in BHK-21 cells. This resulted in a 3-$\log_{10}$ reduction in infectivity and the production of two sizes of DI particles; the larger particle (designated DI-40) was approximately 40% of the length of the B particle, and the smaller particle (designated DI-30) was 30% of the length of the B particle (3).

Quantitation of DI particles. The procedure described by Bellett and Cooper (1) was used to quantitate DI particles. Serial twofold dilutions of a preparation containing DI particles were added to monolayers of BHK-21 cells simultaneously with B particles of VSV_0 (multiplicity of infection [MOI] = 1). The virus inoculum was adsorbed for 1 h at 37 C, and unadsorbed virus was removed by three washes with medium. After overnight incubation, culture fluids were harvested and assayed for VSV infectivity. The interference by DI particles with infectious VSV replication followed a one-particle-per-cell dose response, as has been reported (3, 11).

Complementation tests in BHK-21 cells. Complementation tests were carried out by a modification of the procedure described by Pringle (16). Duplicate monolayers of 5×10^5 to 10^6 BHK-21 cells in 16-mm wells of plastic trays were infected with 0.2 ml of each mutant clone (MOI = 10) or with 0.2 ml of mixtures containing 0.1 ml of each mutant concentration used for single infections. Virus samples were diluted in growth medium plus 10 μg of DEAE-dextran per ml. After an adsorption period of 1 h at 4 C, the inoculum was removed. The monolayers were washed twice with cold medium, 1 ml of warm medium was added, and the trays were transferred in sealed bags (gassed with 5% CO_2 in air) to a water bath at 39.5 $\pm$ 0.05 C. To reduce background infectivity to a minimum, 2 h later the fluids were again removed, the monolayers were washed once with warm medium, 1 ml of warm medium was added, and the plastic trays were returned to the 39.5 C water bath. Six hours later (8 h postinfection), the fluids from the wells were harvested and tested for infectivity at 32 C in CE cell cultures. The results are expressed as a complementation index, which is the ratio: [yield (*m* 1 + *m* 2) at 39.5 C]/[yield *m* 1 at 39.5 C + yield *m* 2 at 39.5 C], where *m* 1 and *m* 2 are a given pair of mutants. Indexes of greater than 2 indicate complementation.

RESULTS

Establishment of persistently infected cell cultures. Our attempts to establish persistent infections in L cells with wild-type VSV (VSV_0) free of DI particles were unsuccessful; at MOIs from 0.1 to 0.00001, all cells were destroyed within a day or two. These results were similar to those reported with BHK-21 cells by Holland and Villarreal (6). However, when we followed their successful technique and infected cells with wild-type B particles in the presence of large numbers of DI particles, persistent infections were readily established. To obtain a suitable mixture of B and DI particles, we used the third undiluted passage of VSV_0 in BHK-21 cells; this pool contained 5.2×10^9 DI and 1.5×10^6 B particles/ml. When monolayer cultures of L cells in 2-ounce bottles (2.5×10^6 cells) were infected with 0.5 ml of this virus pool, the input MOI was 1,000 DI and 0.3 B particles/cell. The virus inoculum was adsorbed for 1.5 h at 37 C and then removed. The cells were washed three times with 5 ml of growth medium, 7 ml of growth medium was added, and the cultures were incubated at 37 C. The medium was removed and replaced with fresh medium 24 h later. Assay of the infectivity of the medium at this time revealed a marked inhibition of virus replication (9.0×10^5 PFU/ml) compared to control cultures infected with a similar low MOI of B particles alone (3.0×10^8 PFU/ml). In addition, there was a marked inhibition of cell destruction (CPE) in the cultures that received the mixture of DI and B particles; only small foci of CPE were observed in cultures inoculated with mixed particles, whereas all cells

were destroyed at 24 h in cultures infected with a similar MOI of B particles alone. Two days after infection, the cells were scraped from the glass and passed to several new bottles. In the succeeding weeks there were frequent crises of cell destruction, followed by regrowth of cells. After many medium changes and cell passages during the next month, the persistently infected cell line stabilized and was maintained in 16-ounce bottles. Confluent cell monolayers were generally formed 4 to 7 days after subculture, and subsequent passages were made by scraping the cells from one culture bottle to three or four new bottles. Trypsin could also be used to detach the cells from the glass. The infected cell line periodically showed areas of cell destruction and significant loss of cell viability. In such cases the cultures were fed frequently with fresh medium until the cells multiplied sufficiently to be passed. There was no regularity in the intervals between crises, and no correlation could be made between the crises and the amount of virus present.

Properties of L cell cultures persistently infected with VSV. When the persistently infected cells (L_{VSV}) had stabilized 6 weeks after the infection was initiated, the virus content of the medium was determined at subsequent cell passages. When assayed at 32 C, the medium always contained between 2×10^4 and 8×10^5 PFU/ml of culture fluid. The characteristics of the virus (VSV_{pi}) present in the persistently infected cells are described in detail in a later section of this paper. Attempts were made to demonstrate the presence of DI particles in the fluids containing plaque-forming virus. The biological interference method for assaying DI particles is described in Materials and Methods. No evidence was obtained for the presence of significant numbers of DI particles in the fluids harvested from L_{VSV} cells. Undiluted infected culture fluids containing 5×10^5 PFU/ml failed to produce any interference with the replication of VSV_0 B particles in either L or BHK-21 cell cultures. To increase the sensitivity of this biological assay for DI particles, 1,600 ml of pooled culture fluids from several consecutive passages of L_{VSV} cell cultures (4.9×10^5 PFU/ml) was concentrated 320-fold by the polyethylene glycol procedure described elsewhere (3, 10). The infectivity titer of the concentrate was 2.3×10^7 PFU/ml, a 47-fold increase over the unconcentrated virus pool. The original pool of VSV_{pi} and the concentrate were tested for their ability to interfere with the replication of VSV_0 in BHK-21 cells. The virus yield (PFU per milliliter) for VSV_0 alone was 8.6×10^8; for VSV_0 plus undiluted original VSV_{pi} pool, 10^9; and for VSV_0 plus undiluted VSV_{pi} concentrate, 1.1×10^9. A significant reduction of VSV yield (approximately 10-fold) would have resulted if, on the average, each cell in the monolayer (2.5×10^6 cells) were infected with 2 DI particles/cell (11). This MOI of DI particles would have occurred if the concentrate had contained 10^7 DI particles/ml (2.5×10^6 cells/culture $\times$ 2 DI/cell, using 0.5 ml of inoculum). Since the concentrate contained 2.3×10^7 PFU/ml and less than 10^7 DI particles/ml by the interference assay, these results suggest that there was less than 1 DI particle/PFU in the fluids harvested from the persistently infected cultures. It should be noted that L cells are poor producers of DI particles during consecutive undiluted passages of VSV_0 (unpublished data). The requirement for large numbers of DI particles per cell to initiate the persistent infection and the presumptive absence of significant numbers of DI particles in the ongoing carrier state will be discussed further below.

The number of L_{VSV} cells plated for each plaque-forming cell was determined by a method described elsewhere (20). In different tests, values form 4 to 100 cells/plaque-forming cell were obtained; this indicated that from 1 to 25% of L_{VSV} cells were capable of forming plaques at a given time. In marked contrast were the results of indirect immunofluorescence tests using rabbit anti-VSV serum and fluorescein-labeled goat anti-rabbit globulin serum. Under these conditions, 100% of acetone-fixed L_{VSV} cells showed heavy cytoplasmic and membrane staining. Uninfected control L cells did not show positive immunofluorescence under the same conditions.

The resistance of L_{VSV} cells to challenge with VSV_0 was determined. L_{VSV} and control L cells were challenged with VSV_0 (MOI = 1). After adsorption for 1 h at 37 C, the monolayers were washed twice and refed with growth medium. After 48 h at 37 C, the control L cells were completely destroyed, whereas the challenged L_{VSV} cells did not differ in appearance from the mock-infected cultures. When assayed at 32 C, VSV_0 plaques (2 to 3 mm) could be readily distinguished from plaques of VSV_{pi} (1 to 1.5 mm). Virus yields in mock-infected and challenged cultures showed that VSV_0 did not replicate in L_{VSV} cells (Table 1). These data, together with the failure of VSV_0 to produce CPE in L_{VSV} cells, indicate that the carried virus establishes strong homologous interference with the replication of wild-type virus.

It was previously reported from this laboratory that VSV completely inhibits the replication of a heterologous herpesvirus, PSR, in a variety of cell lines, including L cells (2, 24). A challenge experiment using PSR was carried

out with L_{VSV} and control L cells in the same manner as described above for challenge with VSV_0. Appropriate antisera and distinctive plaque morphologies were used to differentiate VSV_{pi} and PSR plaques. After incubation of the challenged cultures at 37 C for 48 h, there was a gross difference in the CPE in the two cell lines. Whereas the control L cells showed strong CPE typical of PSR infection, the L_{VSV} cells were unaffected. Titration of the total virus content of the cultures (sonicated cells plus medium) showed that L_{VSV} cells were completely resistant to challenge with PSR (Table 2). This finding is consistent with the observation above that 100% of L_{VSV} cells contain VSV-specific antigen. The specific VSV component responsible for inhibition of PSR replication has not been identified (3). The complete resistance of L_{VSV} cells to PSR is further illustrated by the finding that serial passage of challenged cells can be carried out without the appearance of PSR infectivity or PSR-induced CPE.

No interferon was detected in the culture fluids from L_{VSV} cell cultures. Using methods previously described (20), harvested fluids were dialyzed against glycine-hydrochloride buffer (pH 2.0) overnight at 4 C to destroy VSV_{pi} infectivity, returned to pH 7.0, and then incubated overnight with control L cells. After challenge with VSV_0, no significant inhibition of viral replication was observed. The absence of detectable interferon at early and late passage levels of L_{VSV} cells rules out any role for this mediator in the resistance of these cells to challenge with homologous (VSV_0) and heterologous (PSR) virus. The absence of interferon in L cell cultures persistently infected with a small plaque variant of VSV was also noted by Wagner et al. (21).

TABLE 1. *Superinfection of L_{VSV} and control L cells with wild-type VSV (VSV_0)*

Cells	Virus yield[a] after challenge with:			
	Mock infected		VSV_0 (MOI = 1)	
	PFU/ml[b]	Plaque size (mm)	PFU/ml	Plaque size (mm)
Control L cells	0		1.0×10^8	2-3
L_{VSV} cells[c]	3.1×10^5	<1-1.5	1.8×10^5	<1.-1.5

[a] 48 h at 37 C.
[b] Assays in CE cells at 32 C.
[c] P-50, 310 days after initiation.

Properties of virus (VSV_{pi}) isolated from L_{VSV} cells. When the L_{VSV} line had stabilized about 6 weeks after initiation of the persistent infection, it was noted that the plaque diameter of the carried virus at 32 or 37 C in CE cell cultures was <1 to 1.5 mm after 3 days; after similar incubation, wild-type VSV_0 plaques were 2 to 3 mm in diameter. This change in plaque size is characteristic of virus recovered from other persistent infections, particularly from carrier lines of L cells persistently infected with Newcastle disease virus, as reported from this laboratory previously (20, 23). The plating efficiency of virus from L_{VSV} cultures was determined by carrying out infectivity assays at 32 and 39.5 C; the plating efficiency of VSV_0 was also determined at the two temperatures. Fluids obtained from L_{VSV} cell cultures 52 to 84 days after initiation of the infection showed progressively reduced plating efficiencies at the higher temperature (Table 3). For example, at day 84, the 39.5/32 C ratio was 6.1×10^{-5} compared to a ratio of 0.38 for VSV_0, the wild-type virus. The cutoff temperature for plaque formation by the virus present in the fluids tested (Table 3) was above 37 C; the PFU per milliliter at 32 and 37 C varied within a twofold range. The ts replication of the virus in the 84-day fluid was confirmed by testing the amount of virus produced in CE cells incubated at the permissive and nonpermissive temperatures. Using an MOI of 0.1 (based on the 32 C assay in Table 3), CE cell monolayers were infected with either the 84-day fluid or VSV_0. After virus adsorption for 1 h at 37 C, the cultures were washed twice to remove unadsorbed virus, fresh medium was added, and the cultures

TABLE 2. *Superinfection of L_{VSV} and control L cells with PSR virus*

Cells	Assay[b] with serum	Virus yield[a] after challenge with:			
		Mock infected		PSR (MOI = 1)	
		PFU/ml	Plaque type	PFU/ml	Plaque type
Control L cells	Anti-VSV	0	–	6.5×10^6	PSR
	Anti-PSR	0	–	5.9×10^5	PSR
L_{VSV} cells[c]	Anti-VSV	$<10^1$	–	6.0×10^1	PSR
	Anti-PSR	1.0×10^5	VSV_{pi}	1.2×10^5	VSV_{pi}

[a] 48 h at 37 C.
[b] Assays in CE cells at 37 C.
[c] P-50, 310 days after initiation.

TABLE 3. *EOP at 32 and 39.5 C of fluids from L_{VSV} cell cultures compared with that of wild-type VSV_0*

Test	Days after initiation	Cell passage	Temp (C) of assay	Infectivity in CE cell cultures			
				Medium from L_{VSV} cells		VSV_0 control	
				PFU/ml	39.5/32 C ratio	PFU/ml	39.5/32 C ratio
1	52	P-2	32	2.0×10^5	0.016	8.5×10^6	0.35
			39.5	3.3×10^3		3.0×10^6	
2	63	P-4	32	7.4×10^4	0.058	4.5×10^6	0.44
			39.5	4.3×10^3		2.0×10^6	
3	79	P-6	32	6.2×10^5	$<1.6 \times 10^{-4}$	4.0×10^6	0.35
			39.5	$<10^2$		1.4×10^6	
4	84	P-6	32	6.5×10^5	6.1×10^{-5}	1.5×10^6	0.38
			39.5	4.0×10^1		5.7×10^5	

were shifted to either 32 or 39.5 C and incubated for 48 h. By both CPE and yield determinations, it was confirmed that the virus from L_{VSV} cells was temperature sensitive (Table 4).

The experiments in CE cells described in Tables 3 and 4 were repeated using L cells as the permissive cell; the results obtained were essentially the same. The virus from L_{VSV} cells (84-day fluid) showed a markedly reduced ability to replicate in control L cells at 39.5 C when tested by both the plaquing and yield efficiency methods.

The 84-day L_{VSV} fluid described in Tables 3 and 4 was plated in CE cell cultures at 32 C, and 34 plaques were isolated from terminal dilution plates. The plaque progeny were grown at 32 C in CE cells and tested for efficiency of plating (EOP) at 32 and 39.5 C. The 39.5/32 C EOP for five wild-type clones isolated from VSV_0 ranged from 0.49 to 0.80 (average, 0.67), whereas the ratio for the 34 clones from L_{VSV} cells ranged from 10^{-3} to $<4 \times 10^{-7}$ (average, 7.1×10^{-4}). Clearly, the persistent infection of L_{VSV} cells had spontaneously selected a population of ts viruses. The ts phenotype was stable when clones were passed at low MOI in either L, CE, or BHK-21 cells. A similar selection of stable ts viruses was previously recorded for persistent infections of L, BHK-21, and a line of canine kidney cells with a paramyxovirus, Newcastle disease virus (13, 23).

The RNA phenotype of the 34 ts clones was determined in CE cells at 39.5 C using techniques previously described (12, 13). All 34 clones were defective in RNA synthesis at the nonpermissive temperature: 27/34 synthesized less than 10%, and 7/34 synthesized 10 to 18% of the RNA made at the permissive temperature (32 C). For five clones of wild-type VSV_0, RNA synthesis at 39.5 C ranged from 50 to 91% (average, 60%) of the RNA synthesized at 32 C.

Complementation analyses of ts clones from L_{VSV} cells and known RNA^- mutants representing complementation groups I (ts G 11), II (ts G 22), and IV (ts G 41) of VSV_{IND} were carried out in BHK-21 cells as described in Materials and Methods. Virus pools used for complementation analyses were grown in either CE or BHK-21 cells at 32 C using low MOI (<0.01). Clear evidence of complementation was obtained with all combinations of ts G 11, ts G 22, and ts G 41, which represent known complementation groups (Table 5). Crosses of these mutants with eight of the RNA^- mutants isolated from L_{VSV} cells showed complementation with the group II (ts G 22) and group IV (ts G 41) mutants but not with the group I mutant (ts G 11). When ts pi 26 was crossed in CE cells with the other seven ts pi mutants, the results were negative except for an index of 2.8 in the case of mutant ts pi 3. The degree of complementation seen in the ts pi 26 × ts pi 3 as well as the ts G 11 × ts pi 3 crosses may represent a case of the weak intragenic complementation reported for certain combinations of group I mutants (17). Overall, these results indicate that the eight mutants from the persistent infection that were tested belong to complementation group I. This is not surprising since Flamand (5) reported that 81.7% of spontane-

TABLE 4. *Efficiency of replication at 32 and 39.5 C of fluids from L_{VSV} cell cultures compared with that of wild-type VSV_0*

Inoculum[a]	Temp (C) of incubation	Replication in CE cells		
		CPE at 48 h	Virus yield[b] (PFU/ml)	39.5/32 C ratio
Medium from L_{VSV} P-6 (84 days)	32	++++[c]	1.9×10^8	2.1×10^{-4}
	39.5	0	4.1×10^4	
VSV_0 control	32	++++	1.7×10^8	0.42
	39.5	++++	7.3×10^7	

[a] MOI = 0.1
[b] Assay in CE cells at 32 C.
[c] ++++, Denotes complete cell destruction.

TABLE 5. *Complementation in BHK-21 cells (8 h at 39.5 C)*

Mutant	Complementation index[a]										
	Group			ts pi from L_{vsv} cell line							
	I (ts G 11)	II (ts G 22)	IV (ts G 41)	2	3	9	12	16	18	22	26
ts G 11	(2.3×10^3)[b]	131	265	1.5	2.2	1.9	0.1	0.3	0.1	1.8	0.3
ts G 22	–	(2.5×10^4)	130	72	131	>49	>23	>7	96	16	91
ts G 41	–	–	(3.1×10^4)	2.7	22	11	19	6	32	7	132
ts pi 2				(8.2×10^3)	–	–	–	–	–	–	0.2[c]
ts pi 3					$(<10^2)$	–	–	–	–	–	2.8
ts pi 9						(5.6×10^4)	–	–	–	–	0.6
ts pi 12							(3.7×10^3)	–	–	–	0.5
ts pi 16								(5.3×10^5)	–	–	0.3
ts pi 18									(2.2×10^3)	–	0.5
ts pi 22										(3.0×10^3)	0.3
ts pi 26											(1.1×10^4)

[a] See Materials and Methods for computation.
[b] The values in parentheses are single infection yields expressed as PFU per milliliter.
[c] Crosses of ts pi 26 with the other ts pi clones were done in CE cells.

ous mutants present in VSV_{IND} populations were classed as group I mutants. If the ts mutants that maintain the persistently infected state were originally selected from those spontaneously present in the wild-type population, there would be a strong probability that they would belong to group I. Complementation analyses with additional ts pi mutants are being carried out.

Rate of selection of ts mutants in L cells persistently infected with VSV_0. A prospective study was undertaken to determine more precisely the early events in the establishment of persistent infection, particularly the rate at which ts mutants appeared. This prospective study was started by infecting L cells with the third undiluted passage of VSV_0 in BHK-21 cells, as described above. In this instance, culture fluids were frozen at −70 C at the time of medium changes and at each cell passage. All fluids were tested for plating efficiency in CE cells at 32 and 39.5 C. Selected fluids were then plated in CE cell cultures at 32 C, and isolated plaques were selected at terminal dilutions; 90 clones of the third undiluted passage VSV_0 pool, used to initiate the persistent infection, were also isolated by plating terminal dilutions at 32 C. The ts phenotype of the clones was screened by the CPE method in welled trays at 32 and 39.5 C and then confirmed by the virus yield method, as described in Materials and Methods.

Clonal analysis revealed that the frequency of spontaneous ts mutants in the inoculum used to initiate the persistent infection was 4.4% (Table 6). This frequency of spontaneous ts mutants is roughly comparable to the 2.8% reported for another line of VSV_{IND} (5, 17). Table 6 illustrates that, by 10 days after initiation of the persistent infection, there was a statistically significant increase in the frequency (17.8%) of ts mutants in the fluids harvested from L_{VSV} cells. The frequency of ts mutants increased dramatically in fluids harvested at 11 and 17 days. By 63 days, all 29 clones isolated at 32 C from the L_{VSV} cells were temperature sensitive. The rapid rate of selection of ts mutants by the conditions established in the persistent infection is dramatically illustrated by this prospective study. Possible mechanisms involved in this rapid selection will be discussed below.

It is important to note the limitations of relying on the EOP method alone to determine the presence or absence of ts mutants in a given virus population. It can be noted in Table 6 that a given virus pool (P-2, 11-day fluid) exhibits an EOP (0.18) not significantly different from that of the wild-type virus (0.27); clonal analysis, however, reveals that 73.2% of the clones isolated are ts mutants.

Influence of temperature shift on the characteristics of the L_{VSV} cell line. Replicate cultures of L_{VSV} cells (P-39, 209 days after initiation of the persistent infection) were shifted from 37 C, the usual incubation temperature, to either 32 or 39.5 C. Control cultures were kept at 37 C for comparison. Replicate L_{VSV} cell cultures kept at 37 C did not alter their characteristic pattern of continuous production of virus with a low 39.5/32 C EOP (Table 7, footnote). However, the cultures shifted to 32 C showed significant CPE by 2 days, accompanied by a change in the EOP to a ratio closer to that of the wild type. By 3 days after the shift to

TABLE 6. *Prospective study: appearance of ts mutants in L cells persistently infected with VSV*

Cell passage	Days after initiation	Assay temp (C)	PFU/ml in medium	EOP (39.5/32 C)	No. of clones		% ts	Significance level[a] (*P*)
					Isolated	ts		
(Inoculum)[b]		32	3.0×10^5	0.27	90	4	4.4	
		39.5	8.0×10^4					
P-1	7	32	1.1×10^3	0.55	59	5	8.4	<0.4[c]
		39.5	6.1×10^2					
P-1	10	32	3.9×10^3	0.46	56	10	17.8	<0.025[d]
		39.5	1.8×10^3					
P-2	11	32	3.7×10^5	0.18	56	41	73.2	<0.001[d]
		39.5	6.9×10^4					
P-2	17	32	2.5×10^4	0.088	61	52	85.2	<0.001[d]
		39.5	2.2×10^3					
P-8	63	32	2.2×10^5	0.001	29	29	100	<0.001[d]
		39.5	2.2×10^2					

[a] Student's *t* test.
[b] Third undiluted passage of VSV_0 in BHK cells at 37 C.
[c] Not significant.
[d] Significant.

TABLE 7. *Influence of temperature shift on the characteristics of the persistently infected L_{VSV} cell line*

L_{VSV} cells (P-39, 269 days)[a] shifted from 37 C to:	Cell passage	Days after shift	Assay temp (C)	PFU/ml	39.5/32 C ratio
39.5 C	P-0	1	32	3.1×10^4	2.2×10^{-2}
			39.5	7.1×10^2	
		3	32	2.6×10^4	1.5×10^{-2}
			39.5	3.9×10^2	
	P-1	7	32	4.0×10^2	$<2.5 \times 10^{-2}$
			39.5	$<10^1$	
	P-2	25	32	47	–
			39.5	NT[b]	
		30	32	0	–
			39.5	0	
32 C	P-0	1	32	1.8×10^5	5.0×10^{-3}
			39.5	9.0×10^2	
		2[c]	32	6.5×10^5	0.11
			39.5	7.4×10^4	
		3[d]	32	1.7×10^6	0.11
			39.5	2.0×10^5	

[a] Infectivity of fluids from replicate cultures kept at 37 C for 3 days: 32 C, 2.7×10^5; 39.5 C, 6.2×10^3; 39.5/32 C ratio, 2.2×10^{-2}.

[b] NT, Not tested.

[c] Strong CPE noted.

[d] Cell monolayer completely destroyed.

32 C, there was complete cell destruction and a significant increase in the amount of virus present in the culture fluid.

In marked contrast, the shift of L_{VSV} cells to 39.5 C resulted in continued proliferation of the cells and in a progressively diminished virus production. At 7 days after the shift to 39.5 C, at which time the cells had been passed once at this temperature, there was a 2-$\log_{10}$ reduction in the amount of virus in the medium. The 39.5/32 C EOP ratio of this virus was characteristic of VSV_{pi}. By 25 days (P-2), virus was barely detectable in the medium (47 PFU/ml), and 30 days after the shift-up no detectable virus was present. From this time the cells were passed weekly at 39.5 C without evidence of virus production. The observations described emphasize the role played by ts virus in the maintenance of the persistently infected state.

Shift-down of the "cured" cell line from 39.5 to 32 or 37 C did not result in the reappearance of CPE or infective virus, although the cells were passed weekly at these temperatures. In addition, co-cultivation of the "cured" cells with CE cells for 2 weeks or with BHK-21 cells for seven cell passages over a period of 5 weeks did not result in a reactivation of virus production.

Additional attempts were made to activate virus in the cured line with inhibitors of DNA function or synthesis using a variation of the methods employed by Simpson and Iinuma (18) to activate respiratory syncytial virus in nonproducer, persistently infected lines of bovine embryo kidney cells. Various inhibitors were used to treat producer L_{VSV} lines and a line cured by passage at 39.5 C and maintained at 37 C. The data in Table 8 show that, in producer L_{VSV} cells, only actinomycin D significantly enhanced virus yield 48 h after exposure to the drug was begun. This is not surprising, since VSV replicates independently of endogenous functional host cell DNA. All the other inhibitors used failed to alter significantly the virus yield per cell in the producer L_{VSV} line. In the case of the cured line, no activation of virus replication occurred with any of the inhibitors used (Table 8). This pattern of behavior after treatment with DNA inhibitors is in contrast to the report by Simpson and Iinuma that halogenated pyrimidines activated production of infective virus in nonproducer lines of bovine embryo kidney cells persistently infected with respiratory syncytial virus (18).

TABLE 8. *Treatment of producer and cured lines of L_{VSV} with DNA inhibitors: influence on virus production*

Treatment of cells[a]	Virus yield expressed as PFU/ml (PFU/cell) from:	
	L_{VSV} cells, producer line (P-60, 344 days at 37 C)	Cured L_{VSV} cells[b]
None	3.3×10^4 (0.028)	$<10^1$ (0)
Actinomycin D (2 μg/ml)	1.2×10^5 (2.00)	$<10^1$ (0)
Mitomycin C (0.5 μg/ml)	3.1×10^4 (0.026)	$<10^1$ (0)
Bromodeoxyuridine (100 μg/ml)	1.3×10^5 (0.11)	$<10^1$ (0)
Cytosine arabinonucleoside (10^{-5} M)	3.1×10^4 (0.062)	$<10^1$ (0)

[a] Cell monolayers in 60-mm dishes were exposed to the inhibitors in medium containing 4% dialyzed calf serum for 20 hr at 37 C. The drug was removed, and the cells were washed three times with warm medium and refed with medium +4% dialyzed calf serum. Culture fluid was harvested 48 h after treatment with the inhibitor was begun, and the number of cells in each dish was determined. The infectivity of the fluids was tested in CE cell monolayers at 32 C. PFU per cell was determined by dividing the cell number per dish by the total PFU present in 3 ml of culture fluid.

[b] Cured L_{VSV} cells: P-39 (269 days at 37 C) shifted to 39.5 C became negative for virus after 30 days at 39.5 C (Table 7); shifted to 37 C after an additional 21 days at 39.5 C; treated with drugs after 59 days (P-8) at 37 C.

Ability of ts mutants to establish persistent infection. An attempt was made to establish a persistent infection in L cells using a cloned ts mutant derived from L_{VSV} cells. To this end, clone ts pi 364, which was isolated from the 7-day fluid of the persistent infection summarized in Table 6, was used. The original plaque isolate grown at 32 C was used to initiate the infection; this plaque isolate contained 8.4×10^7 PFU/ml and showed a 39.5/32 C yield efficiency of $<1.1 \times 10^{-6}$. L cell monolayers in 2-ounce bottles (3.4×10^6 cells) were infected with 0.5 ml of 10-fold dilutions of ts pi 364 to give input MOIs ranging from 0.1 to 0.00001. These low MOIs and the nature of the initial plaque isolate would preclude a role for DI particles in the initiation of the carrier state. After an adsorption period of 1 h at 37 C, the monolayers were washed twice with growth medium, 7 ml of fresh medium was added, and the cultures were incubated at 37 C. By 4 days, the cultures infected at the three highest MOIs showed complete cell destruction and virus yields were considerable, although the progeny were still clearly ts (Table 9). The two lowest MOIs (0.0001 and 0.00001) produced only several small foci of cell destruction, and the fluids from these cultures contained reduced amounts of ts virus (Table 9). The two persistently infected L_{VSV} lines established using the lowest MOI of ts virus were passed at 4- to 6-day intervals. At this writing these lines have been maintained at 37 C for eight cell passages over a period of 43 days. Only occasional foci of cell damage have been seen, and the infectivity of the medium ranges from 10^3 to 10^5 PFU/ml when assayed in CE cell cultures at 32 C. The 39.5/32 C EOP ratio has not exceeded 2.2 $\times$ 10^{-4}, indicating the persistence of a ts virus population in the carrier cultures. Continued passage and characterization of this cell line is being carried out.

TABLE 9. *Attempt to initiate persistent infection of L cells at 37 C using different MOIs of clone ts pi 364: cytopathology and virus replication 4 days after initiation*

Input MOI	CPE (4 days at 37 C)	Infectivity of medium 4 days after infection		
		Temp (C) of assay[a]	PFU/ml	39.5/32 C ratio
0.1	++++[b]	32	9.3×10^6	5.6×10^{-5}
		39.5	5.3×10^2	
0.01	++++	32	$>5.0 \times 10^6$	–
		39.5	$>1.0 \times 10^2$	
0.001	++++	32	$>5.0 \times 10^6$	$<1.0 \times 10^{-5}$
		39.5	$<1.0 \times 10^1$	
0.0001	±	32	2.2×10^6	$<4.5 \times 10^{-5}$
		39.5	$<1.0 \times 10^1$	
0.00001	±	32	8.0×10^5	$<1.2 \times 10^{-5}$
		39.5	$<1.0 \times 10^1$	

[a] Assays in CE cell monolayers.
[b] ++++, Denotes complete cell destruction.

The ts pi 364 pool used to initiate the persistent infection described above was tested for its ability to replicate at 32, 37, and 39.5 C. When the EOP at these temperatures was compared (Table 10) to that of a wild-type clone (WT_02), it was found that there was a marked decrease in the ability of ts pi 364 to replicate at 37 C, compared to its replication at 32 C. At 39.5 C the conditions were nonpermissive, providing a 39.5/32 C ratio of 1.4×10^{-5}. This "leakiness" of ts pi 364 at 37 C is an important aspect of the mechanism for establishment and maintenance of persistent infection by ts mutants, which is proposed in the Discussion.

DISCUSSION

The results presented demonstrate the important role played by ts virus in the establishment and maintenance of persistent infections of L cells with VSV. For clarity, it is desirable to separate this process into two components, establishment and maintenance, and to examine what is known about each.

The establishment of persistent infection with VSV at 37 C in L cells depends on the characteristics of the virus used. For example, when wild-type infectious B particles are used, it is necessary to coinfect the cells with large numbers of DI particles to establish the carrier state. Even at extremely low MOIs, B particles alone replicate and completely destroy the host cells. These findings are in agreement with those of Holland and Villarreal (6), who used VSV_{IND} and BHK-21 cells. However, the requirement for DI particles to establish persistent VSV infections in L cells can be eliminated by initiating the infection with a very low MOI of a ts mutant. A clonal isolate of mutant ts pi 364, which had been obtained from L_{VSV} cells, was capable of establishing a stable carrier state when used at an MOI of 10^{-4} or 10^{-5}. Under these conditions, the persistent infection was initiated with ts virus propagated and diluted to exclude DI particles. In addition, low MOI precluded coinfection of any cells with both a B and DI particle, if present. It is apparent from the results that the establishment of persistent infection of L cells with VSV can occur without a requirement for mediation by DI particles. The ability of ts virus at low MOI to establish the carrier state may depend on the decreased efficiency of this virus to replicate in L cells at 37 C. In effect, replication of ts virus at this temperature may be leaky, leading to an

TABLE 10. *EOP of ts pi 364 in CE cell cultures at 32, 37, and 39.5 C compared with that of a VSV_0 clone (WT_02)*

Virus clone	Assay temp (C)	PFU/ml[a]	Plaque size (mm)	EOP ratio		
				37/32 C	39/37 C	39/32 C
WT_02	32	3.9×10^8	5-6			
	37	4.2×10^8	5-6	1.07	0.80	0.87
	39.5	3.4×10^8	3-4			
ts pi 364[b]	32	1.2×10^8	1-2			
	37	2.3×10^5	<1-1	1.9×10^{-3}	7.3×10^{-3}	1.4×10^{-5}
	39.5	1.7×10^3	<1			

[a] After 4 days at specified temperature.
[b] Original plaque isolate from L_{VSV} 7 days after initiation, as described in Table 6.

altered virus-host cell interaction. This interpretation is in agreement with the report by Farmilo and Stanners (4) that hamster embryo cells infected at 38.5 C with ts T 1026 (a group I mutant of the HR strain of VSV_{IND} isolated in Toronto) were able to continue to synthesize DNA and undergo as many as 10 divisions at 38.5 C without cytocidal effects. This situation was considered to be a consequence of the semipermissive conditions used in their experiments. On the other hand, when wild-type virus or large amounts of ts virus are used at 37 C, DI particles are essential to reduce the initial virus replication and cell destruction that follow. Only by the intercession of DI particles can the destructive effects of the virus be modulated sufficiently for persistence to follow under these conditions.

In regard to maintenance of the persistently infected state, the question again arises concerning the role of DI particles. Efforts to demonstrate the presence of DI particles in the L_{VSV} cell line were not successful. Using a biological interference assay in BHK-21 cells, it was concluded that there was less than 1 DI particle/PFU in the fluids harvested from L_{VSV} cell cultures. Even concentration of the infected fluid failed to reveal the presence of significant numbers of DI particles. The presence of low levels of DI particles might be revealed by using the amplification methods suggested by Holland and Villarreal (7), and efforts in this direction are continuing. However, there is other indirect evidence that reduces the possiblility that DI particles are required for maintenance of the carrier state in L_{VSV} cells. When the persistently infected cells maintained at 37 C are shifted down to 32 C, there is an increased replication of virus, and complete cell destruction occurs within 3 days. Holland and Villarreal (6) also reported a rapid cytocidal effect accompanied by virus replication when their line of BHK-21 cells persistently infected with VSV was shifted down from 37 to 33 C. It is difficult to reconcile these findings with maintenance of the carrier state by the presence of DI particles. There is no evidence in the literature and no a priori reason to believe that DI particles show a temperature-dependent interference effect (8).

The selection of a ts virus population occurs with surprising rapidity when L cells are infected with low multiplicities (0.3 PFU/cell) of wild-type B particles in the presence of large numbers of DI particles (1,000/cell). In one experiment (Table 6) in which a detailed prospective study was undertaken, at 10 days there was an increase to 17.8% in the frequency of ts clones in the virus population. By 17 days, this frequency had reached 85.2%, and by 63 days after the initiation of the infection 100% of the clones isolated had a ts phenotype. This persistently infected L_{VSV} cell line has been maintained for 7 months without the appearance of revertants in the virus population.

When L_{VSV} cells passed at 37 C were treated with several inhibitors of DNA synthesis or function, only actinomycin D produced a significant change in virus yield, i.e., a 100-fold increase (Table 8). This result, together with the failure of mitomycin C, bromodeoxyuridine, or cytosine arabinonucleoside to affect virus yields, indicates that it is unlikely that a DNA provirus state is involved in regulating virus production in this persistent infection, as has been suggested for other systems by several groups of workers (18, 25, 26). Additional presumptive evidence against the involvement of a DNA provirus in the L_{VSV} cell line was provided by data described in Table 8. A line of L_{VSV} cells was converted to a nonproducer line by shift-up to and passage at 39.5 C for 30 days. When this cured cell line was shifted back to 37 C, no virus replication was detected. Treatment of the cured cell line with the DNA inhibitors

listed above failed to activate virus production. Attempts to find evidence for a DNA provirus are continuing, using more sensitive hybridization techniques that employ purified L_{VSV} cell DNA and isotope-labeled virion RNA from VSV.

Questions that remain unanswered by the data provided in this paper are: "How are ts mutants able to establish and maintain persistent infections?" and "Why don't revertants replace the ts population at 37 C, a temperature not optimum for the ts mutants?" A possible answer to these questions may be provided by preliminary evidence that known RNA^- mutants belonging to VSV complementation groups I, II, and IV, as well as a group I mutant (ts pi 26) isolated from L_{VSV} cells, are capable of interfering with the replication of wild-type VSV in control L and BHK-21 cells. We have previously reported that ts mutants of Newcastle disease virus interfere with wild-type virus replication at both permissive and nonpermissive temperatures (13). This interference was prior to or at the level of RNA transcription. It has also been reported by Stollar et al. (19) that ts Sindbis virus interferes with wild-type virus replication in BHK-21 cells. In addition, we have found that under conditions of mixed infections at 37 and 39.5 C the ts mutants are "rescued" by the wild-type virus. ts virus yields are dramatically increased, while, at the same time, the wild-type virus replication is suppressed significantly at both temperatures. This "dominance" of the replication of ts virus at 37 C provides a rationale for the spontaneous selection and maintenance of ts mutants in the persistently infected cell lines. Details of these experiments will be published separately.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-06264 from the National Institute of Allergy and Infectious Diseases.

The help with statistical analyses by Georg Keleti and the assistance of Evelyn Ketz are gratefully acknowledged.

LITERATURE CITED

1. **Bellett, A. J. D., and P. D. Cooper.** 1959. Some properties of the transmissible interfering component of vesicular stomatitis virus preparations. J. Gen. Microbiol. **21**:498-509.
2. **Dubovi, E. J., and J. S. Youngner.** 1976. Inhibition of pseudorabies virus replication by vesicular stomatitis virus. I. Activity of infectious and inactivated B particles. J. Virol. **18**:526-533.
3. **Dubovi, E. J., and J. S. Youngner.** 1976. Inhibition of pseudorabies virus replication by vesicular stomatitis virus. II. Activity of defective interfering particles. J. Virol. **18**:534-541.
4. **Farmilo, A. J., and C. P. Stanners.** 1972. Mutant of vesicular stomatitis virus which allows deoxyribonucleic acid synthesis and division in cells synthesizing viral ribonucleic acid. J. Virol. **10**:605-613.
5. **Flamand, A.** 1970. Etude génétique du virus de la stomatite vésiculaire: classement de mutants thermosensibles spontanés en groups de complémentation. J. Gen. Virol. **8**:187-195.
6. **Holland, J. J., and L. P. Villarreal.** 1974. Persistent noncytocidal vesicular stomatitis virus infections mediated by defective T particles that suppress virion transcriptase. Proc. Natl. Acad. Sci. U.S.A. **71**:2956-2960.
7. **Holland, J. J., and L. P. Villarreal.** 1975. Purification of defective interfering T particles of vesicular stomatitis and rabies viruses generated *in vivo* in brains of newborn mice. Virology **67**:438-449.
8. **Huang, A. S.** 1973. Defective interfering viruses. Annu. Rev. Microbiol. **27**:101-117.
9. **Huang, A. S., and D. Baltimore.** 1970. Defective viral particles and viral disease processes. Nature (London) **226**:325-327.
10. **McSharry, J. J., and R. Benzinger.** 1970. Concentration and purification of vesicular stomatitis virus by polyethylene glycol precipitation. Virology **40**:745-779.
11. **Marcus, P. I., and M. J. Sekellick.** 1974. Cell killing by viruses. I. Comparison of cell-killing, plaque formation and defective-interfering particles of vesicular stomatitis virus. Virology **57**:321-338.
12. **Preble, O. T., and J. S. Youngner.** 1972. Temperature-sensitive mutants isolated from L cells persistently infected with Newcastle disease virus. J. Virol. **9**:200-206.
13. **Preble, O. T., and J. S. Youngner.** 1973. Temperature-sensitive defect of mutants isolated from L cells persistently infected with Newcastle disease virus. J. Virol. **12**:472-480.
14. **Preble, O. T., and J. S. Youngner.** 1973. Selection of temperature-sensitive mutants during persistent infection: role in maintenance of persistent Newcastle disease virus infections of L cells. J. Virol. **12**:481-491.
15. **Preble, O. T., and J. S. Youngner.** 1975. Temperature-sensitive viruses and the etiology of chronic and inapparent infections. J. Infect. Dis. **131**:467-473.
16. **Pringle, C. R.** 1970. Genetic characteristics of conditional lethal mutants of vesicular stomatitis virus induced by 5-fluorouracil, 5-azacytidine, and ethyl methane sulphonate. J. Virol. **5**:559-567.
17. **Pringle, C. R.** 1975. Conditional lethal mutants of vesicular stomatitis virus. Curr. Top. Microbiol. Immunol. **69**:85-116.
18. **Simpson, R. W., and M. Iinuma.** 1975. Recovery of infectious proviral DNA from mammalian cells infected with respiratory syncytial virus. Proc. Natl. Acad. Sci. U.S.A. **72**:3230-3234.
19. **Stollar, V., J. Peleg, and T. E. Shenk.** 1974. Temperature sensitivity of a Sindbis virus mutant isolated from persistently infected *Aedes aegypti* cell culture. Intervirology **2**:337-344.
20. **Thacore, H. R., and J. S. Youngner.** 1969. Cells persistently infected with Newcastle disease virus. I. Properties of mutants isolated from persistently infected L cells. J. Virol. **4**:244-251.
21. **Wagner, R. R., A. H. Levy, R. M. Snyder, G. A. Ratcliff, Jr., and D. F. Hyatt.** 1963. Biologic properties of two plaque variants of vesicular stomatitis virus (Indiana serotype). J. Immunol. **91**:112-122.
22. **Wertz, G. W., and J. S. Youngner.** 1972. Inhibition of protein synthesis in L cells infected with vesicular stomatitis virus. J. Virol. **9**:85-89.
23. **Youngner, J. S., and D. O. Quagliana.** 1975. Temperature-sensitive mutants isolated from hamster and canine cell lines persistently infected with Newcastle disease virus. J. Virol. **16**:1332-1336.
24. **Youngner, J. S., H. R. Thacore, and M. Kelly.** 1972.

Sensitivity of ribonucleic and deoxyribonucleic acid viruses to different species of interferon in cell cultures. J. Virol. 10:171-178.

25. **Zhdanov, V. M., N. N. Bogornolova, V. I. Garilov, O. G. Andzhaparidze, R. G. Zmieva, and A. N. Astakhova.** 1974. Infectious DNA of tickborne encephalitis virus. Arch. Gesamte Virusforsch. 45:215-224.
26. **Zhdanov, V. M., and M. I. Parfanovich.** 1974. Integration of measles virus nucleic acid into the cell genome. Arch. Gesamte Virusforch. 45:225-234.