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Persistent Infection of L Cells with Vesicular Stomatitis Virus: Evolution of Virus Populations

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A previous report (Youngner et al., J. Virol. **19**:90-101, 1976) documented that noncytocidal persistent infection can be established with wild-type vesicular stomatitis virus (VSV) in mouse L cells at 37°C and that a rapid selection of RNA^-, group I temperature-sensitive (*ts*) mutants consistently occurs in this system. To assess the selective advantage of the RNA^- *ts* phenotype, evolution of the virus population was studied in persistent infections initiated in L cells by use of VSV *ts* 0 23 and *ts* 0 45, RNA^+ mutants belonging to complementation groups III and V. In L cells persistently infected with *ts* 0 23, the *ts* RNA^+ virus population was replaced gradually by viruses which had a *ts* RNA^- phenotype. VSV *ts* 0 45 (V) has another marker in addition to reduced virus yield at 39.5°C: a defective protein (G) which renders virion infectivity heat labile at 50°C. Persistent infections initiated with this virus (*ts*, heat labile, RNA^+) evolved into a virus population which was *ts*, heat resistant, and RNA^-. These findings suggest that the *ts* phenotype itself is not sufficient to stabilize the VSV population in persistently infected L cells and also indicate that the *ts* RNA^- phenotype may have a unique selective advantage in this system. In addition to the selection of *ts* RNA^- mutants, other mechanisms which also might operate in the maintenance of persistent VSV infections of L cells were explored. Whereas defective-interfering particles did not seem to mediate the carrier state, evidence was obtained that interferon may play a role in the regulation of persistent infections of L cells with VSV.

In 1976, we reported (16) that noncytocidal persistent infections could be established in mouse L cells (L_{VSV}) at 37°C with infective B particles of vesicular stomatitis virus (VSV) in the presence of large numbers of defective-interfering (DI) particles. Within a few weeks after infection, there was a rapid spontaneous selection of temperature-sensitive (*ts*) virus which replaced the wild-type virus population. A significant rise in the frequency (73%) of *ts* mutants in the virus population was noted by 11 days, and by 63 days 100% of the virus clones isolated were *ts* at the nonpermissive temperature (39.5°C). The *ts* clones isolated had an RNA^- phenotype and belonged to VSV complementation group I. This finding was so consistent in persistent infections initiated in L cells with wild-type VSV that the possible selective advantage of the RNA^- phenotype in this system had to be considered.

The present report describes the evolution that occurs in virus populations when persistent VSV infections of L cells are established with phenotypically RNA^+ mutants belonging to complementation groups III and V. In addition, an assessment is made of several mechanisms which may operate in the maintenance of persistent infections by VSV in L 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 *ts* mutants of VSV_{IND} used represented complementation groups III and V and were obtained from R. R. Wagner. Stocks of virus were grown in BHK-21 cells with the use of inocula of less than 0.01 PFU/cell to minimize the production of DI particles. Infectivity assays were done in CE cells.

Screening of VSV clones for temperature sensitivity. CE monolayers were grown at 37°C in plastic trays containing 24 wells 16 mm in diameter. Each well was seeded with 1.5 ml of a standard dilution of primary CE cells; from 8×10^5 to 1×10^6 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 suspensions obtained by transferring agar over isolated plaques to 1.0 ml of medium. Replicate well trays were incubated at 33 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 33°C (usually 48 h after infection), fluids were harvested from wells at both temperatures and assayed by the plaque technique at 33°C.

VSV-specific RNA synthesis. Cumulative VSV-specific RNA synthesis in BHK-21 cells was determined as follows. BHK-21 cells in plastic well trays (24 wells, 16 mm in diameter) were infected (multiplicity of infection [MOI] = 5) for 1 h at 4°C. The virus diluent contained DEAE-dextran (10 μg/ml). The inoculum was removed, 1 ml of warm growth medium was added, and duplicate trays were incubated at either 33 or 39.5°C for 1 h. The medium was removed, the monolayers were washed with 1 ml of warm growth medium, and 1 ml of warm growth medium containing 4 μg of actinomycin D per ml and 4 μCi of [5-^{3}H]-uridine (specific activity, 29 Ci/mmol) was added. The duplicate trays were incubated in water baths (± 0.05°C) at either 33 or 39.5°C for 8 h. The medium was removed, the cells were washed twice with cold medium, and the monolayers were solubilized with 1% (wt/vol) sodium dodecyl sulfate in phosphate-buffered saline lacking Ca^{2+} and Mg^{2+}. A 2-ml amount of cold 10% trichloroacetic acid was added, and the trichloroacetic acid-insoluble fraction was collected after 1 h at 4°C by suction filtration on Whatman GF/A glass-fiber filters. The filters were oven-dried (80°C for 20 min) and treated with 0.5 ml of NCS tissue solubilizer (Amersham/Searle Corp.) for 1 to 2 h at room temperature. Radioactivity was determined in a Packard 3375 liquid scintillation spectrometer after adding 10 ml of a toluene-based scintillation cocktail containing 42 ml of Liquifluor (New England Nuclear Corp.) per liter of toluene. Clones that synthesized RNA with an efficiency ratio less than two-tenths of the 39.5°C to 33°C ratio of RNA synthesis by wild-type VSV were classed as having an RNA$^-$ phenotype. This method of analysis was used to obviate the reduced efficiency of RNA synthesis by many *ts* viruses at the permissive temperature as a factor in the analysis (2).

RESULTS

Initiation of persistent infection with a group III *ts* mutant (*ts* 0 23). To investigate the possible selective advantage of RNA$^-$ VSV mutants in persistence, infections were initiated with an RNA$^+$ mutant (*ts* 0 23) belonging to complementation group III. Persistence was established at 37°C in L cells infected with a low multiplicity (0.4) of this virus. The inoculum had been grown in BHK-21 cells at low MOI to minimize the presence of DI particles. Fluids were collected from the persistently infected line at the time of each cell passage. At selected intervals after initiation of the persistence, the virus in harvested fluids was cloned in CE cells by isolating plaques at terminal dilutions at 33°C, the permissive temperature. In addition, the parental virus was cloned to determine its homogeneity in regard to the RNA$^+$ phenotype. The results of these cloning experiments are displayed in Table 1.

Clonal analysis revealed that in the case of the *ts* 0 23 parental population, 29 of 30 clones (97%) showed an RNA$^+$ phenotype; one clone synthesized RNA at the nonpermissive temperature with an effectiveness only 10% of that of wild-type virus. By 2 days after initiation of the infection, the frequency of RNA$^-$ mutants present in the fluids from persistently infected cells had risen to 10%, an increase not statistically significant. However, at 37 days, the frequency of RNA$^-$ mutants was 68%; at 75 and 198 days after initiation, the frequency of RNA$^-$ mutants increased to 72% and 81%, respectively. The results obtained with individual clones, displayed graphically in Fig. 1, reveal that, in addition to the increasing frequency of RNA$^-$ clones in the population, the RNA synthesis defect became progressively more pronounced with time.

To confirm these results, another persistent infection was started in L cells by use of a subclone (F-3) of *ts* 0 23 that had been plaque-purified six times in CE cells. The sixth clonal passage was amplified at low multiplicity of infection (0.01) in MDBK cells, which are poor producers of DI particles. When clonal analysis techniques were applied to fluids harvested at different times after initiation of persistence, the pattern observed was similar to that of cultures infected with parental *ts* 0 23 as described above (Fig. 2). The RNA synthesis at the nonpermissive temperature of the parental clone F-3 was as efficient as or more so than the wild-type virus and is shown by the solid square symbols at the top of Fig. 2. At 8 days after initiation, all 30 clones tested were *ts* and 29 of 30 clones were RNA$^+$ at the nonpermissive temperature; one clone was classed as RNA$^-$. By 43 days, all 29

TABLE 1. *Appearance of RNA$^-$ phenotype in persistent infections of L cells initiated with a group III (RNA$^+$) ts mutant of VSV (ts 0 23)*

Cell passage	Days after initiation	No. of clones *ts*/no. isolated	No. of clones with RNA$^-$ phenotype[a]
Parental virus (*ts* 0 23)	—	30/30	1 (3)
P-0	2	30/30	3 (10)
P-8	37	28/28	19 (68)
P-18	75	29/29	21 (72)
P-44	198	26/26	21 (81)

[a] RNA$^-$ phenotype = 39.5°C to 33°C ratio of RNA synthesis in BHK-21 cells by the cloned virus divided by the 39.5°C to 33°C ratio of wild-type virus in infected cells is 0.2 or less. Numbers within parentheses indicate percentages.

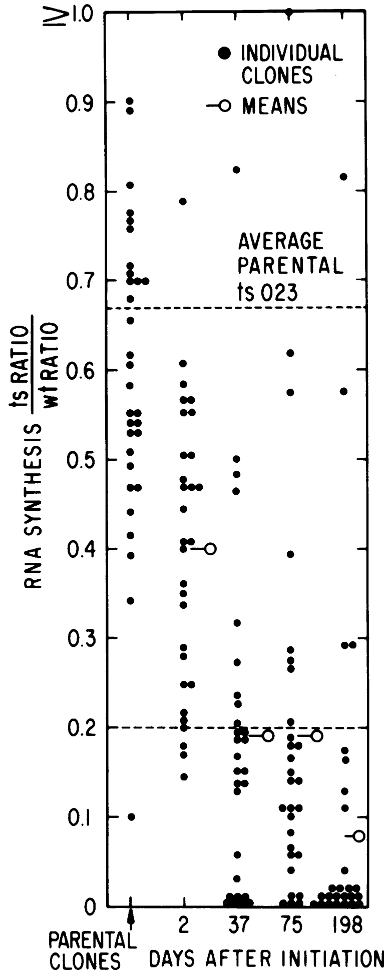

FIG. 1. *Efficiency of RNA synthesis (39.5°C to 33°C ratio) of ts virus clones isolated at different intervals from persistent infection of L cells initiated with ts 0 23, a group III RNA+ mutant of VSV. Time on abscissa not to scale.*

clones isolated were still *ts* but, of these, 23 (79%) were RNA−. At 211 days after initiation, of 28 *ts* clones isolated, 27 (96%) had an RNA− phenotype. Again it can be noted, as in Fig. 1, that in addition to the increasing frequency of RNA− clones in the population, the RNA synthesis defect became progressively more pronounced with time.

Attempts were made to carry out complementation analysis of the RNA− clones isolated from the persistent infections initiated with *ts* 0 23; these efforts were unsuccessful. Problems were encountered involving the leakiness of these mutants at 39.5°C when used at high multiplicities in complementation analyses. Clones with 39.5°C/33°C plating efficiencies of 10^{-5} to 10^{-6} gave yield efficiencies of 10^{-1} to 10^{-2} at a high (5.0) MOI. This is in marked contrast to the behavior of the *ts* mutants from persistent infections initiated in L cells with wild-type VSV (16). Schnitzer et al. (12) have reported that MOI influences the leakiness of the *ts*-1 mutant of respiratory syncytial virus at 39°C.

Initiation of persistent infection with a group V *ts* mutant (*ts* 0 45). In addition to reduced viral replication at 39.5°C, *ts* 0 45 has another phenotypic marker: the G protein is defective and renders the virion infectivity heat labile at 50°C (9). This marker enabled further characterization of the evolution of virus in persistent infections with this group V mutant.

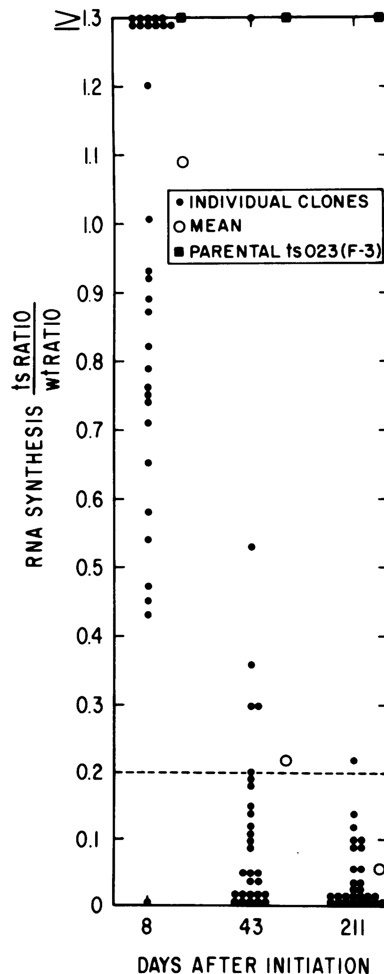

FIG. 2. *Persistent infection of L cells initiated with a six times cloned VSV group III RNA+ mutant (ts 0 23 F-3): efficiency of RNA synthesis (39.5°C to 33°C ratio) of ts virus clones isolated at different times after initiation. Time on abscissa not to scale.*

The *ts* 0 45 virus used for initiation had been serially cloned six times in CE cells and then amplified in MDBK cells at low MOI to minimize the production of DI particles. Persistent infection was established by use of an input multiplicity of 0.01. Samples of the culture fluids were harvested at various intervals after initiation, and the efficiency of plating (EOP) at 39.5°C was determined. Individual virus clones were isolated from terminal dilution plates incubated at 33°C. The properties of clones obtained from fluids harvested between 7 and 235 days after initiation are summarized in Table 2. Although infectivity was barely detectable in fluids harvested during the first two passages, titers subsequently fluctuated between 10^3 and 10^5 PFU/ml. No correlation was apparent between the properties of the individual virus clones isolated and the infectivity titer in the culture fluids or the extent of cytopathic effect in the cultures. However, at 16 and 21 days after initiation, the high EOP at 39.5°C of the uncloned culture fluids was indicative of the presence of a large number of non-*ts* (ts^+), apparently wild-type revertant clones in the virus population. The ts^+ clones replicated to high titers in CE and BHK-21 cells at both 33 and 39.5°C and were somewhat more heat resistant than authentic wild-type virus. The reasons for the appearance of wild-type revertants at this time and for their subsequent elimination from the virus population are unclear. By 36 days, the virus population was again predominantly (85%) *ts*, and from 49 days on all virus clones isolated were *ts*. Concurrent with these changes in the virus population, the heat lability of the *ts* virus clones changed dramatically. Figure 3 displays graphically the effect of heating on the infectivity of clones isolated at different times after initiation of persistence with *ts* 0 45. The first panel of clones at the left of Fig. 3 shows the data for parental clones, all of which were extremely heat labile at 50°C. At 7 days after

TABLE 2. *Evolution of virus in L cells persistently infected with VSV ts 0 45 (V)*

Cell passage	Days after initiation	EOP (39.5/33°C)	No. of clones *ts*/no. isolated	Percent *ts*
P-1	7	ND[a]	15/15	100
P-3	16	0.27	11/30	37
P-4	21	0.18	5/30	17
P-7	36	$<1.3 \times 10^{-3}$	23/27	85
P-9	49	$<6.6 \times 10^{-4}$	30/30	100
P-11	79	$<3.1 \times 10^{-3}$	30/30	100
P-16	118	9.1×10^{-3}	28/28	100
P-44	235	$<9.0 \times 10^{-5}$	30/30	100

[a] Not determined.

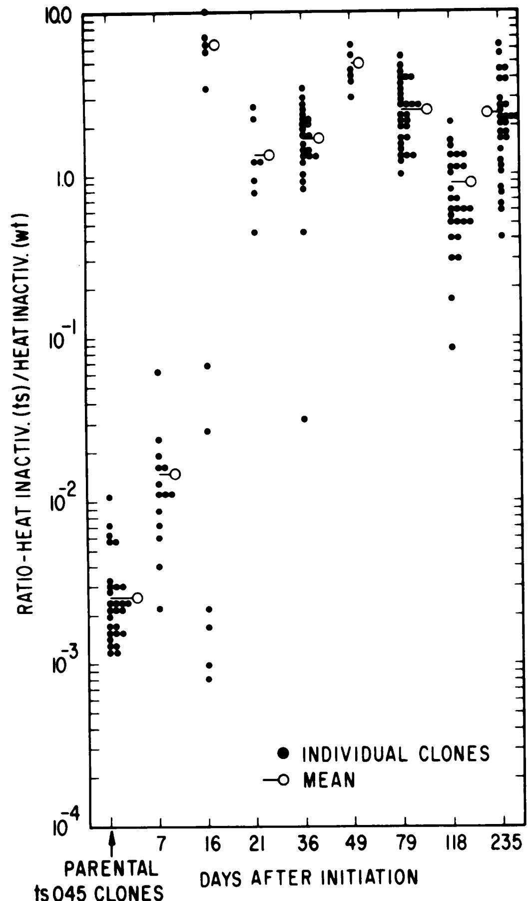

FIG. 3. *Evolution of ts virus clones isolated at different intervals from persistent infection of L cells with ts 0 45 (F-4), a six times cloned VSV group V RNA⁺ mutant. Inactivation of infectivity (compared to wild-type virus) after heating at 50°C for 10 min. Virus samples were diluted 1:100 in phosphate-buffered saline (pH 7.2) prior to heating.*

initiation of infection, all 15 virus clones isolated were *ts*, and all 14 of those tested at 50°C were heat labile, with heat inactivation ratios ranging from 6×10^{-2} to 2×10^{-3}, relative to wild-type VSV. This range was very similar to that found for the clones of parental mutant *ts* 0 45. In contrast, the 11 *ts* clones isolated 16 days after initiation of the persistence fell into two groups: six clones (*ts* hl [heat labile]) were similar to the parental *ts* 0 45 virus, whereas five clones (*ts* hr [heat resistant]) were 3.5- to 10-fold more heat resistant than wild-type VSV. All *ts* virus clones isolated from subsequent passages of the persistently infected cells, with only one exception (see 36-day clones in Fig. 3), were *ts* hr.

The *ts* virus clones from 16-, 79-, and 118-day fluids failed to give a consistent complementa-

tion pattern with any of the known genetic groups of VSV. Most of the virus clones isolated 21 days or later after initiation produced unacceptably high yields under the conditions of high MOI required for either complementation analyses or cumulative RNA synthesis measurements at 39.5°C. When clonal fluids of 21- or 36-day clones were used to infect CE, L, or BHK-21 cells at 33 and 39.5°C, all clones were uniformly *ts* and had replication efficiencies between 10^{-3} and $<10^{-6}$. However, the 33°C yield from this first amplification passage in either CE or BHK-21 cells had plaquing efficiencies at 39.5°C between 10^{-1} and 10^{-3}, indicating the accumulation of ts^+ revertants during the passage at 33°C. The high reversion frequency effect was much more pronounced when CE or BHK cells were infected at an MOI of 5 than at an MOI of 0.05 and was termed "multiplicity leak." However, it can be noted in the last column of Table 3 that, of 26 stable *ts* hr clones (i.e., clones which did not appear to accumulate revertants) isolated between 16 and 235 days after initiation, 22 (84.6%) were RNA^-.

From the data in Fig. 3 and Table 3, the following picture emerges. During the course of establishment and maintenance of persistent infection initiated with a group V mutant in L cells, the uniformly *ts* hl RNA^+ virus population present 7 days after infection evolved by 2 to 3 weeks into a mixture in which ts^+ virus predominated. The apparently wild-type virus (ts^+ hr RNA^+) was then rapidly replaced by a *ts* hr RNA^- population, suggesting that the pressures within the persistent infection select specifically for certain types of *ts* mutants most suited for maintaining the carrier state.

TABLE 3. *Heat sensitivity and RNA phenotype of virus clones isolated from persistent infection initiated with ts 0 45*

Days after initiation	*ts* Heat-sensitive clones		*ts* Heat-resistant clones	
	No.	RNA^+/RNA^- [a]	No.	RNA^+/RNA^- [a]
Parental virus (*ts* 0 45)	28	28/0 [b]	0	—
7	14	13/1	0	—
16	6	5/0	5	1/3
21	0	—	7	ML [c]
36	1	ML [c]	24	0/1
49	0	—	30	ML [c]
79	0	—	30	2/4
118	0	—	28	0/8
235	0	—	29	1/6

[a] RNA phenotype of stable *ts* persistently infected clones only.

[b] An additional 27 parental *ts* 0 45 F-4 clones (not tested for heat sensitivity marker) were tested for RNA phenotype; 3 of 27 clones were RNA^-.

[c] Multiplicity leak.

Mediation by DI particles. We reported previously that noncytocidal persistent infections of L cells could be established with wild-type VSV only in the presence of large numbers of DI particles (16). Although essential for initiation, DI particles were not considered crucial to the maintenance of this persistent infection since we failed to demonstrate significant numbers of DI particles in L_{VSV} fluids (less than one DI particle/PFU) by an interference assay (1). Further efforts were made to quantitate the DI particles present in culture fluids by using the highly sensitive amplification technique of Holland and Villarreal (4). Using this method and employing BHK-21 cells for amplification, we found less than one DI particle for each 10 PFU in the culture fluids from L_{VSV} cell cultures. In addition, serial undiluted passages in BHK-21 cells of fluids from L_{VSV} cell cultures showed that at least three passages were necessary before interference and DI bands were detected in the harvested fluids, again indicating the low concentration of DI particles in the fluids from L_{VSV} cell cultures. We have also found that, compared to BHK-21 cells, L cells are poor hosts for the replication of VSV DI particles (unpublished data). The deficient replication of VSV DI particles in L cells has also been noted by others (8, 11).

As Holland et al. (5) have pointed out, it is practically impossible to rule out the presence of small numbers of DI particles in VSV replicating systems. In the case of L_{VSV} cells, the paucity of demonstrable DI particles in the culture fluids and the inherent defectiveness of the host L cells for their production make it seem unlikely that, in the system we are studying, DI particles play a crucial role in the maintenance of the carrier state.

Mediation by interferon. In our original studies with L_{VSV}, we reported that although the cells were resistant to challenge with wild-type VSV and a heterologous virus, pseudorabies, no interferon was detected in the culture fluids, even after 100-fold concentration (16). More recently, Ramseur and Friedman reported that prolonged VSV infections of L cells, established with the aid of exogenous interferon, may be maintained by endogenous interferon production as well as by the selection of *ts* mutants (10, 11). In their experiments, endogenous interferon was implicated not by the detection of the inhibitor in fluids from the infected cells (7) but by the enhancement of virus replication and cell destruction after treatment of the cell cultures

with anti-mouse interferon antibody. This approach had previously been used by Inglot et al. (6) to elucidate the role of endogenous interferon in persistent infections of L cells with Sindbis virus.

Cell lines persistently infected with VSV were treated with a 1:100 dilution of rabbit anti-mouse interferon immunoglobulin G (supplied by the Antiviral Substances Program, National Institute of Allergy and Infectious Diseases), with normal immunoglobulin G, or with growth medium alone. The results obtained with two L_{VSV} cell lines (Table 4) show that in both instances continued presence of anti-interferon antibody in the medium increased virus titers 34- to 180-fold in fluids harvested 3 and 6 days after treatment was begun. Correlated with the increased virus yield, there was also a significant increase in cell destruction in both cell lines when treated with anti-interferon antibody. These results, which are quite similar to those obtained by Ramseur and Friedman (10, 11), point to the possible role of endogenous interferon production in the maintenance of persistent VSV infections in L cells. It should be noted that these cell lines are resistant to challenge not only with VSV, but also with such heterologous viruses as pseudorabies, Mengo, and encephalomyocarditis. In addition, only a minority (1 to 12%) of the persistently infected cells give evidence of infection with VSV when tested by plating of infected cells or by immunofluorescence. This evidence is also compatible with a role for endogenous interferon in the maintenance of persistence in these cells.

The evidence, albeit indirect, that interferon activity is present in L_{VSV} cell cultures suggested the possibility that this inhibitory state could exert pressure for the selection of *ts* mutants during viral persistence (11). To test this possibility, six times cloned wild-type VSV was serially passed at low MOI in L cells which had been pretreated for 20 h with L-cell interferon induced by Newcastle disease virus infection (18). Six serial passages were made of virus harvested from interferon-treated cultures which displayed a yield reduction of 10^{-2} to 10^{-4} compared to yields in untreated L cells. Six parallel serial passages of the wild-type VSV were made in L cells treated with medium alone. After the sixth serial passage, the 39.5°C/33°C plating efficiency of the virus harvested from interferon-treated or control cell cultures was not different from the plating efficiency of the wild-type virus inoculum. To check more thoroughly for the presence of *ts* mutants in the various populations, the inoculum and the viruses recovered from six serial passages in either interferon or medium were cloned, and each clone was screened by the well-tray method for *ts* phenotype. It can be noted in Table 5 that six serial passages at low MOI in L cells pretreated with interferon did not increase the frequency of *ts* virus above that spontaneously present in the wild-type inoculum.

TABLE 4. *Influence of anti-mouse interferon antibody on virus yield and cell destruction in L_{VSV} cell lines*

L_{VSV} cell line	Duration of treatment (days)	Infectivity of culture fluid (PFU/ml) from cells treated with:		
		Minimal essential medium	Rabbit immunoglobulin G[a]	
			Normal	Anti-mouse interferon
402 (P-17, 70 days)	3	1.1×10^4 (0)[b]	1.6×10^4 (0)	3.0×10^6 (++++)
312 (P-115, 547 days)	3[c]	1.0×10^4 (±)		4.5×10^5 (±)
	6	5.3×10^4 (±)		1.8×10^6 (++++)

[a] At a 1:100 dilution.
[b] (0) = No cell damage visible; (±) = traces of cell damage; (++++) = complete cell destruction.
[c] Cells split 1:2.

DISCUSSION

The data presented demonstrate that, when persistent infections are initiated in L cells with RNA^+ VSV mutants representing complementation groups III and V, the viruses which evolve are quite different from the parental virus. While there is a long-term maintenance of the *ts* marker, a selection of viruses with RNA^- phenotype occurs. These findings suggest that the *ts* phenotype itself is not sufficient to stabilize the virus population in this persistent infection. If it were sufficient, then the infections initiated with *ts* RNA^+ mutants would have continued to produce virus with *ts* RNA^+ phenotypes. Instead, in L cells persistently infected with VSV

TABLE 5. *Serial passage of wild-type VSV in L cells pretreated with interferon: influence on frequency of ts mutants in the virus population*

Wild-type VSV passage history	Clones picked	Clones *ts*	Percent *ts*
Inoculum[a]	19	1	5.2
Passed six times in medium	20	0	0.0
Passed six times in medium + interferon[b]	40	1	2.5

[a] Amplified pool of sixth clonal passage.
[b] Yield reduction at each passage 1.8×10^{-2} to 7.6×10^{-4}.

there seems to be a strong selective advantage for RNA⁻ viruses. This selection was first noted in our earlier experiments in which persistent infection of L cells was established with wild-type VSV in the presence of large numbers of DI particles (16). In this instance there was a rapid selection of *ts* RNA⁻ viruses which replaced the wild-type parent (*ts*⁺ RNA⁺) used to initiate the carrier culture. The *ts* RNA⁻ virus clones isolated from persistent infections initiated with wild-type virus belonged to VSV complementation group I; the defect in this complementation group is in the L protein, the virion-associated transcriptase. To date we have not been able to analyze by complementation the RNA⁻ mutants from persistent infections initiated with *ts* RNA⁺ viruses. In addition to a high degree of leakiness when these mutants are used at the high multiplicities required for complementation analyses, the RNA⁻ clones appear to have multiple mutations. Continued efforts are being made to utilize other techniques to identify the nature of the RNA⁻ defect in these clones, e.g., complementation techniques which utilize low MOI and direct analysis of the *ts* defect in RNA synthesis (transcription versus replication of RNA).

The lack of selective advantage of a "late" defect in a structural protein is illustrated by the results obtained in the case of persistent infection established with *ts* 0 45, a group V mutant. In addition to reduced virus yields at the nonpermissive temperature (39.5°C), this RNA⁺ mutant has an additional phenotypic marker: the virion G protein is defective and renders the virus infectivity heat labile at 50°C. Comparison was made of the heat stability of *ts* clones isolated from the persistent infection, of the *ts* 0 45 parental virus, and of wild-type VSV. This analysis revealed that the heat lability due to a defective G protein was lost rather soon after the establishment of the carrier infection. The *ts* hl RNA⁺ parental *ts* 0 45 inoculum evolved into a virus population that was *ts* hr RNA⁻. On the basis of this finding, it appears that a defect in the G protein provides no selective advantage during persistence in L cells, in contrast to the RNA⁻ phenotype, which does. However, we have not yet tested the heat resistance of *ts*⁺ revertants of parental *ts* 0 45 to determine whether conversion from hl to hr always coincides with loss of the G protein defect.

Consideration must be given to the question: why does the RNA⁻ phenotype confer a selective advantage to the virus which evolves in the persistent infection? We pointed out earlier (16) that the *ts* RNA⁻ clones recovered from L_{VSV} cells replicate significantly less efficiently at 37°C than they do at 32 or 33°C. Furthermore, we suggested that the maintenance of the carrier state may depend on the decreased efficiency of replication of *ts* RNA⁻ virus in L cells at 37°C. The replication of *ts* RNA⁻ virus at this temperature is leaky, and this may lead to an altered virus-host interaction. Virus with the RNA⁻ phenotype would transcribe less RNA at 37°C and thereby reduce the amount of translation of virion proteins which are responsible for shut-off of host macromolecular synthesis. As a consequence, there would be a reduction at 37°C of the cytopathic effects of virus infection, allowing persistently infected cells to survive.

Another question to be considered is the origin of the *ts* RNA⁻ VSV mutants which appear in persistent infections initiated with wild-type VSV plus DI particles (16) or with *ts* RNA⁺ mutants belonging to complementation groups III and V. Clonal analysis of wild-type VSV (16) and group III and V *ts* RNA⁺ mutant populations used to initiate persistent infections in L cells revealed the presence of RNA⁻ viruses at a frequency ranging from 3 to 6%. It is possible that the *ts* RNA⁻ viruses which eventually predominate in the persistent infection are selected from those present in the original parental population. Since about 80% of spontaneous *ts* mutants of VSV map in group I (3), it is also possible that the occasional *ts* mutants which arise spontaneously during virus replication are amplified during subsequent growth cycles as a result of their reduced cytopathogenicity and, therefore, greater selective advantage. Studies are in progress to determine whether the early RNA⁻ mutation is in addition to, and masks, the original RNA⁺ defect, or whether completely "new" mutants are selected.

Our investigations of a number of mechanisms that may operate in the maintenance of persistent VSV infections of L cells revealed that the production of endogenous interferon may play a role. Treatment of persistently infected cells with rabbit anti-mouse interferon immunoglobulin G caused a significant increase in both virus production and cell destruction. From indirect evidence we concluded that this endogenous interferon production did not exert pressure for the selection of *ts* mutants. Despite the apparent presence of endogenous interferon in persistently infected L cells, there are still some unresolved questions this observation raises. If interferon is mediating the persistently infected state, why do the cells maintained at 37°C show high virus yields and undergo cell destruction when shifted down to 32 or 33°C (10, 16)? Why is it impossible to establish prolonged VSV infections in L cells pretreated with interferon at

37°C and then infected and shifted down to 32°C (11)?

In addition to the studies summarized above, Sekellick and Marcus (13) have proposed that activation of the interferon system by the [±] RNA class of DI particles may provide the means by which persistently infected cells survive in the presence of otherwise lethal virus. However, it is difficult to invoke the interferon mechanism in the case of cell lines which are defective IF producers. Despite this deficiency, these cell lines (Vero or BHK-21) have been persistently infected with a variety of viruses (14, 15, 17). It seems likely that a multiplicity of factors acting in concert or individually may be responsible for the establishment and maintenance of persistent infections with a variety of viruses in different cell lines.

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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. **Bratt, M. A., and L. E. Hightower.** 1977. Genetics and paragenetic phenomena of paramyxoviruses, p. 457–533. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 9. Plenum Press, New York.
3. **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.
4. **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.
5. **Holland, J. J., L. P. Villarreal, and M. Breindl.** 1976. Factors involved in the generation and replication of rhabdovirus defective T particles. J. Virol. **17**:805–815.
6. **Inglot, A. D., M. Albin, and T. Chudzio.** 1973. Persistent infection of mouse cells with Sindbis virus: role of virulence of strains, auto-interfering particles and interferon. J. Gen. Virol. **20**:105–110.
7. **Nishiyama, Y.** 1977. Studies of L cells persistently infected with VSV: factors involved in the regulation of persistent infection. J. Gen. Virol. **35**:265–279.
8. **Potter, K. N., and R. B. Stewart.** 1976. Comparison of vesicular stomatitis virus defective interfering particle synthesis in chick embryo and L cells. Can. J. Microbiol. **22**:1458–1463.
9. **Pringle, C. R.** 1977. Genetics of rhabdoviruses, p. 239–289. *In* H. Fraenkel-Conrat and R. R. Wagner (ed.), Comprehensive virology, vol. 9. Plenum Press, New York.
10. **Ramseur, J. M., and R. M. Friedman.** 1977. Prolonged infection of interferon-treated cells by vesicular stomatitis virus: possible role of temperature-sensitive mutants and interferon. J. Gen. Virol. **37**:523–533.
11. **Ramseur, J. M., and R. M. Friedman.** 1978. Prolonged infection of L cells with vesicular stomatitis virus. Defective interfering forms and temperature-sensitive mutants as factors in the infection. Virology **85**:253–261.
12. **Schnitzer, T. J., L. S. Richardson, and R. M. Chanock.** 1976. Growth and genetic stability of the *ts*-1 mutant of respiratory syncytial virus at restrictive temperatures. J. Virol. **17**:431–438.
13. **Sekellick, M. J., and P. I. Marcus.** 1978. Persistent infection. I. Interferon-inducing defective-interfering particles as mediators of cell sparing: possible role in persistent infection by vesicular stomatitis virus. Virology **85**:175–186.
14. **Stanwick, T. L., and J. V. Hallum.** 1974. Role of interferon in six cell lines persistently infected with rubella virus. Infect. Immun. **10**:810–815.
15. **ter Meulen, V., and S. J. Martin.** 1976. Genesis and maintenance of a persistent infection by canine distemper virus. J. Gen. Virol. **32**:431–440.
16. **Youngner, J. S., E. J. Dubovi, D. O. Quagliana, M. Kelly, and O. T. Preble.** 1976. Role of temperature-sensitive mutants in persistent infections initiated with vesicular stomatitis virus. J. Virol. **19**:90–101.
17. **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.
18. **Youngner, J. S., A. W. Scott, J. V. Hallum, and W. R. Stinebring.** 1966. Interferon production by inactivated Newcastle disease virus in cell cultures and in mice. J. Bacteriol. **92**:862–868.