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Viruses Isolated from Cells Persistently Infected with Vesicular Stomatitis Virus Show Altered Interactions with Defective Interfering Particles

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Virus mutants isolated from persistent infections of vesicular stomatitis virus in BHK-21 cells were much less susceptible to interference mediated by the defective interfering particle used to establish the persistent infection. This mutational change occurred as early as 34 days in the persistent infection and continued for over 5 years. The earliest variants showed no oligonucleotide map changes and no difference in the temperature-sensitive phenotype from the original virus, but the later variants exhibited extensive map changes. These results suggest a possible role for defective interfering particles in the selection of the mutants.

A number of animal viruses which are normally cytocidal are capable of establishing a long-term persistent infection in cultured cells. Although the precise mechanisms responsible for the establishment and maintenance of persistent infections of RNA viruses have not been clearly defined, a number of factors have been implicated: (i) temperature-sensitive mutants (18, 27), (ii) interferon production (14, 15, 19), and (iii) defective interfering (DI) particles (1, 5, 10, 17, 20, 22, 26). Each of these factors may play a greater or lesser role depending on the virus-cell system studied.

Huang and Baltimore (7) first suggested that DI particles might play an important role in viral persistence. The best-characterized persistent infection in which DI particles play a major role is that of vesicular stomatitis virus (VSV) in BHK-21 cells (5, 6). DI particles were necessary for both the establishment and maintenance of the persistent infection (5). The BHK-21 CAR4 persistently infected cell line has been maintained for 6.5 years and continues to shed low levels of standard virus and a constantly changing population of DI particles. Standard virus isolated from the persistent infection at various times was analyzed by T_1 oligonucleotide mapping of the viral RNA, and it was shown that the virus shed by this culture is undergoing extensive and continuous mutational change (4). Mutations are exhibited in all five viral proteins and in the ends of the viral RNA (21, 23). The recovered viruses exhibit a small-plaque phenotype; they have become increasingly less cytopathic and can establish a persistent infection after 69 months without added DI particles (6, 21). Little is known about the selective mechanisms involved in promoting such a rapid mutation rate, and it is difficult to determine which mutations have biological importance since the mutational evolution of the virus is both extensive and continuous.

Many investigators have obtained mutations in the virus isolated from persistent infections in other systems (1, 12, 13, 18, 24, 25). These viruses often exhibit a small-plaque or temperature-sensitive phenotype. Of particular interest was the finding by Kawai and Matsumoto (9) that a small-plaque variant of rabies virus isolated from a persistent infection was less susceptible to interference by the original DI particle used to establish this persistent infection. They proposed that this small-plaque variant could be effectively selected since it is much less susceptible to interference by the preexisting DI particles.

We report here a similar finding in the case of persistent infections of VSV in BHK-21 cells and present a quantitative preliminary characterization of these viruses.

The persistently infected line studied here (designated CAR4) was originally established by coinfection of BHK-21 cells with a high multiplicity of the cloned *ts*G31 mutant of the VSV Indiana serotype and its homologous DI particle (5). After establishment of the persistent infection at 33°C, the cells were maintained at 37°C. Virus was isolated at various times from the persistently infected culture by cocultivation of carrier cells and normal BHK-21 cells. Virus was plaque purified, and high-titer clonal pools were prepared for use in the subsequent experiments.

We then examined the susceptibility of these viruses to interference mediated by the original

DI particle used to establish this persistent infection (*ts*G31 DI). We also quantitated the ability of the recovered viruses to replicate this DI particle. This was done by coinfecting identical monolayers of BHK-21 cells with the infectious virus at a multiplicity of 5 PFU/cell together with increasing amounts of the purified *ts*G31 DI particle. Interference was quantitated by plaque assay of the yield. Replication of the DI particle was examined after velocity gradient centrifugation of progeny virus on sucrose gradients to separate standard virus and DI particles. We quantitated the DI particle yield spectrophotometrically as described in the legend to Fig. 1. A typical sucrose gradient profile is shown in Fig. 1a. The results are shown in Fig. 1b and c and summarized in Table 1. The mixed population of virus isolated from the persistent infection at 75 days contained 70% small plaques (1 to 2 mm). The remainder of the plaques were of a size comparable to the *ts*G31 standard virus (4 mm). The small-plaque phenotype bred true, and no detectable interference or DI particle replication was observed even at a level of input DI particles which gives over 1 log interference with the homologous standard virus. The large-plaque virus at 75 days postinfection (75-day LP) was still subject to *ts*G31 DI particle-mediated interference and still replicated that DI particle, although some quantitative differences were observed. At 14 months, the virus isolated from the persistent infection was of large-plaque phenotype and was once again sensitive to *ts*G31 DI particle-mediated interference. In contrast, the small-plaque virus isolated at 5 years was insensitive to these levels of the *ts*G31 DI particle. For each virus examined, the amount of interference observed was directly related to the level of DI particle replication (compare Fig. 1b and c). This correlation shows that either assay can be quantitatively used to study DI particle-mediated interference.

To determine how quickly this mutational change can occur in the standard virus during persistent infection, a separate carrier line (designated CAR21) was established in BHK-21 cells by the method by which the CAR4 persistent infection had been established, and with the exception that these carrier cells were maintained at 33°C at all times (5). Virus was isolated by direct plaque purification from the supernatant of the persistent infection. Figure 1d and e shows that this mutation which confers a relative resistance to the original DI particle can take place by 34 days, at which time a similar mixed population of small-plaque (34-day SP) and large-plaque (34-day LP) virus was found. The 34-day SP virus was not sensitive to interference mediated by the *ts*G31 DI particles at the levels of input DI particles employed in this experiment. However, the virus isolated at 24 days in this persistent infection was entirely large plaque and showed normal levels of interference by the *ts*G31 DI particle (Table 1).

We next determined whether extremely high multiplicities of the *ts*G31 DI particle could interfere well with the 34-day SP and 75-day SP viruses. Detectable levels of DI particle replication and interference did occur with these viruses at an input of 10 DI units per culture, and greater than 90% interference was obtained when 100 DI units of the *ts*G31 particle were used. However, we observed that the *ts*G31 DI particle always depressed the yield of homologous *ts*G31 virus at least 10- to 1,000-fold greater than it depressed the yield of 34-day SP and 75-day SP viruses, even under the extreme interference conditions of high input DI particles. Similar results were seen if a low multiplicity of infection (0.01 PFU/cell) of standard virus was employed with a high input of *ts*G31 DI particles, and the virus yield was assayed during the first cycle of infection (at 9 h postinfection). Clearly the mutant viruses are much more resistant to the original DI particle, but this resistance is quantitative and not absolute.

We next carried out reciprocal experiments to determine whether the original standard virus (*ts*G31) is subject to interference by DI particles generated by the virus variants isolated after various periods of persistent infection. BHK-21 cells were coinfected with a high multiplicity (5 PFU/cell) of standard virus and 5 to 10 DI units of each DI particle studied. Table 1 shows that greater than 90% interference occurred with the original *ts*G31 standard virus production when 5 to 10 DI units of either the 34-day SP DI particle or the 5-year DI particle was used. It is evident from these results that this mutational change is unidirectional. Standard virus underwent a mutational change which rendered it less sensitive to the original DI particle, whereas DI particles generated by these viruses remained potent interfering agents against the original standard virus.

It was of interest to determine whether these mutations which confer resistance to the *ts*G31 DI particle always cause a detectable T_1 oligonucleotide map change in viral RNA. Cloned virus was labeled with ^{32}P and purified. The viral RNA was isolated and digested with T_1 nuclease, and the resulting oligonucleotides were mapped on two-dimensional gels as previously described (4). The oligonucleotide maps of the large- and small-plaque viruses isolated at 75 days from CAR4 and 34 days from CAR21 are identical to

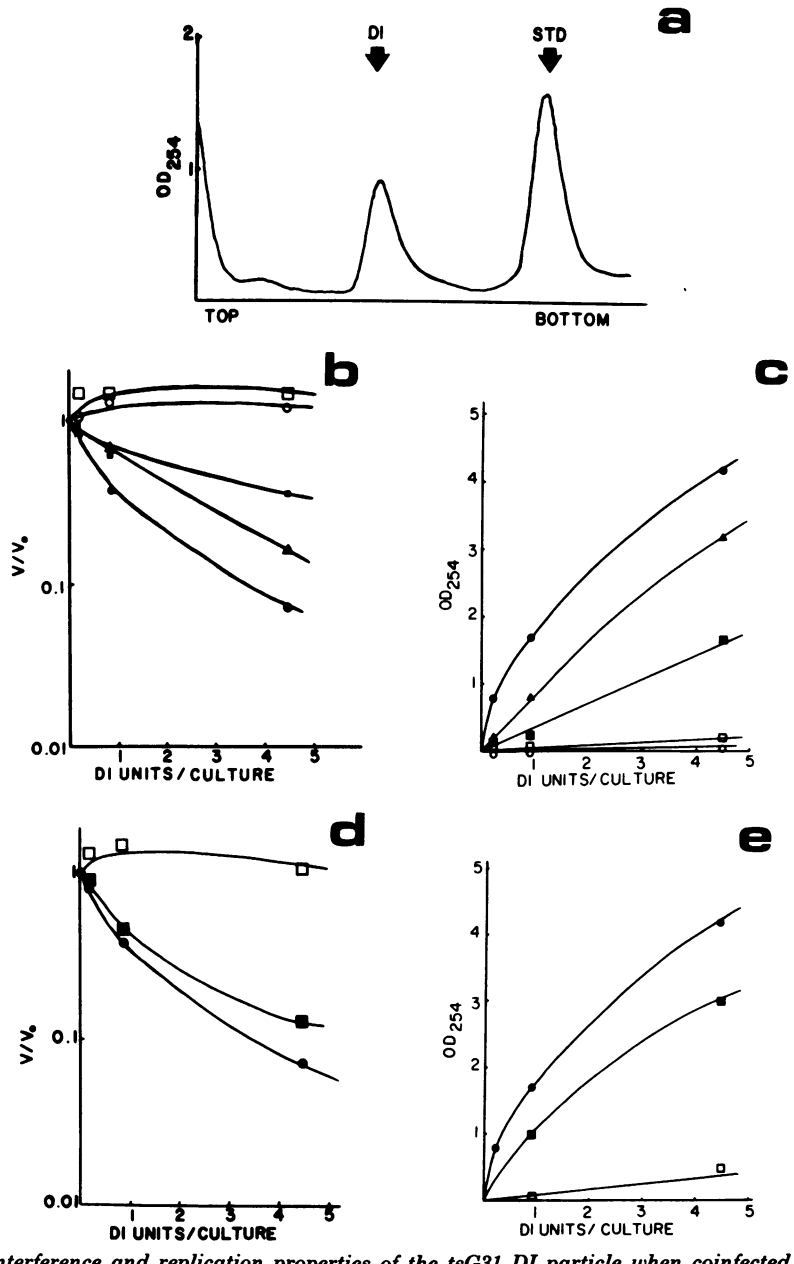

FIG. 1. *Interference and replication properties of the tsG31 DI particle when coinfected with standard viruses isolated from CAR4 (b and c) and CAR21 (d and e). Matched monolayers of* 4×10^7 *BHK-21 cells were coinfected with cloned standard virus at a multiplicity of infection of 5 PFU/cell and tsG31 DI particles which were purified by two successive centrifugations in a 5 to 40% (wt/vol) sucrose gradient. Since these viruses are temperature sensitive, all infections and assays were done at 33°C. This DI particle purification procedure reduced the level of contaminating standard virus to less than 1 particle per* 10^8 *(3). Culture fluids were harvested at 48 h postinfection and assayed for infectivity by plaque assay. Progeny virus was pelleted and centrifuged on a 5 to 40% (wt/vol) sucrose gradient (3). The gradients were fractionated continuously from the top through an ISCO model UA-5 absorbance monitor which measures absorbance at 254 nm, and the DI particle yields were quantitated by weighing the peaks. A typical sucrose gradient is shown in (a). V, PFU per milliliter after addition of various doses of DI particles;* V_0 *PFU per milliliter without addition of DI particles. (b and c) ●, tsG31; □, 75-day SP; ■, 75-day LP; ▲, 14 month; ○, 5 year. (d and e) ●, tsG31; □, 34-day SP; ■, 34-day LP.*

TABLE 1. *Summary of interference properties of standard virus and DI particles isolated from persistent infection*[a]

Cell line	Standard	DI particle	Interference (90% or greater)
CAR4	*ts*G31	*ts*G31	+
	75-day SP	*ts*G31	−
	75-day LP	*ts*G31	+
	14 month	*ts*G31	+
	5 year	*ts*G31	−
	*ts*G31	5 yr	+
	5 yr	5 yr	+
CAR21	*ts*G31	*ts*G31	+
	24 day	*ts*G31	+
	34-day SP	*ts*G31	−
	34-day LP	*ts*G31	+
	*ts*G31	34-day SP	+
	34-day SP	34-day SP	+
	34-day LP	34-day SP	+

[a] Monolayers of BHK-21 cells were coinfected with standard virus at a multiplicity of infection of 5 PFU/cell and 5 to 10 DI units of purified DI particles. One DI unit is defined as the number of DI particles in the inoculum which gave 37% of the yield of DI particle-free inocula (i.e., which gave one hit biologically [2]).

the original *ts*G31 virus (data not shown). It is not surprising that these mutations are undetectable by T_1 oligonucleotide mapping of the viral RNA, since only about 10% of all mutations result in alterations in the unique oligonucleotides.

We next determined whether the temperature-sensitive phenotype of the recovered viruses had been altered during the course of the persistent infection. The yield of these viruses grown at 33, 37, and 39°C was determined (Table 2). All of the viruses recovered from the persistent infection were still temperature sensitive as evidenced by at least a 2-log reduction in yield when the viruses were grown at 39°C as compared with the yield when the viruses were grown at 33°C.

There are undoubtedly many selective pressures exerted on the virus during a persistent infection, and DI particles appear to provide one type of selective pressure. The virus, once selected for greater resistance to the original DI particle population can then generate DI particles which can interfere with the replication of the newly selected virus as well as the original viruses still present in the population. The population of DI particles present has been shown to change during the course of the persistent infection (5). We do not yet know whether newly arising DI particles in persistent infections can select for multiple rounds of mutations in a similar manner or whether this selection only occurs once, soon after the establishment of the persistent infection. We are carrying out further isolation of viruses from CAR4 and CAR21 and testing their susceptibility to interference mediated by these DI particles to resolve this question.

We have recently obtained some evidence that DI particles might be involved in the selection of mutations. We repeatedly passaged standard virus in BHK-21 cells without dilution (to ensure that high levels of DI particles are present during each serial passage). These serial undiluted passages appear to be generating mutations detectable by T_1 oligonucleotide fingerprinting at a much faster rate than passage either at high dilution or alternating high and low dilutions (4, 21; K. Spindler, unpublished data). DI particles may be partly involved directly by selection effects and perhaps indirectly by slowing virus replication rates and prolonging cell survival times. However, many other factors in high-multiplicity passages (such as complementation among multiple mutant viruses in the same cell) may play a role. J. S. Youngner, E. V. Jones, M. Kelley, and D. W. Frielle (submitted for publication) have observed a striking increase in the percentage of temperature-sensitive mutants of VSV present in the virus population after 4 to 10 high-multiplicity passages in L cells. However, they did not observe this in BHK-21 cells unless they pretreated the cells with actinomycin D, and they could not correlate the increase in temperature-sensitive mutants with DI particle production. They postulate the involvement of host cell fidelity factors. Further work will be needed to assess the influence of DI particles on accumulation of mutations in virus populations.

The location of the sites of mutation on the viral genome which confer a relative resistance

TABLE 2. *Growth characteristics of viruses isolated from persistent infection*[a]

Virus	Yield at 37°C/yield at 33°C	Yield at 39°C/yield at 33°C
*ts*31	0.24	0.00025
34-day SP	1.0	0.0093
34-day LP	0.04	0.00025
75-day SP	0.50	0.0014
75-day LP	0.84	0.010
MSB (wild type)	0.69	0.11

[a] Monolayers of BHK-21 cells were infected at a multiplicity of infection of 5 PFU/cell. Infections were carried out at 33, 37, and 39°C. Virus yield was determined by plaque assay at 33°C.

to DI particles should shed some light on those regions of the viral RNA which play a role in autointerference. It has been suggested that the complementary terminal sequences present in the DI particle RNA may contribute to the replicative advantage of the DI RNA during an infection (8, 16). Since the 5′ end of the viral RNA is conserved in the RNA of the DI particle (11, 16), and its complement is substituted at the 3′ end, both of these sequences would be reflected in the 5′ end of the viral RNA. It has been shown that the 5′ end of the viral RNA mutates much more rapidly than does the 3′ end during persistence (21, 23). The virus variants described here may result from a coordinated coselection of mutations in the 5′ end of the viral RNA and in the gene(s) coding for the replicase (and possibly other gene products). Sequencing and hybridization studies to test the importance of these regions of the genome are being initiated.

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