Reconstruction Experiments Demonstrating Selective Effects of Defective Interfering Particles on Mixed Populations of Vesicular Stomatitis Virus

By F. M. HORODYSKI† AND J. J. HOLLAND*
Department of Biology, C-016, University of California, San Diego, La Jolla, California 92093, U.S.A.

(Accepted 26 January 1984)

SUMMARY

Mutants of vesicular stomatitis virus have previously been isolated whose replication is not affected by the presence of defective interfering (DI) particles. We describe here reconstruction experiments in BHK-21 cells which show that DI particle-resistant viruses are strongly selected over wild-type virus in the presence of DI particles. This occurs both during undiluted lytic passage and persistent infection. This selection is evident even when the mutant virus is added initially at very low levels relative to wild-type virus. This indicates that DI particles can be important selective factors affecting viral evolution.

RNA viruses exhibit very high mutation frequencies (for review, see Holland et al., 1982). RNA virus populations include numerous different virus variants but an equilibrium is usually reached in which the fastest-replicating (or most fit) virus variant predominates (Domingo et al., 1978). Vesicular stomatitis virus (VSV) maintains a rather stable equilibrium under dilute passage conditions since no oligonucleotide map changes are observed under these conditions on repeated passage (Clewley et al., 1977; Freeman et al., 1979; Holland et al., 1979; Rowlands et al., 1980; Spindler et al., 1982). However, mutants of a variety of RNA viruses accumulate rapidly during persistent infections (Wagner et al., 1963; Mudd et al., 1973; Preble & Youngner, 1973; Kawai et al., 1975; Ahmed & Graham, 1977; Holland et al., 1979; Weiss et al., 1980; Meinkoth & Kennedy, 1980), and during serial high multiplicity (undiluted) passages in cell culture (Ahmed et al., 1980; Brand & Palese, 1980; Youngner et al., 1981; Spindler et al., 1982).

Defective interfering (DI) particles accumulate during undiluted passages (Huang & Baltimore, 1970), and they are required for many types of persistent RNA virus infections (Holland & Villarreal, 1974; Kawai et al., 1975; Ahmed & Graham, 1977; Popescu & Lehmann-Grube, 1977; Roux & Holland, 1979; Weiss et al., 1980; McCarthy et al., 1981; Andzhapidze et al., 1982). The presence of DI particles may be one of the factors disturbing virus population equilibria during persistence or high multiplicity infections because virus mutants resistant to DI particles (S di− mutants) can arise (Kawai & Matsumoto, 1977; Horodyski & Holland, 1980; Jacobsen & Pfau, 1980; Horodyski et al., 1983). These resistant virus mutants can subsequently generate new DI particles able to interfere with the S di− virus (Kawai & Matsumoto, 1977; Horodyski & Holland, 1980), until new S di− variants arise which escape interference by the new DI particles.

To determine directly whether DI particles can disturb the dynamic equilibrium of virus populations, we performed reconstruction experiments in which we infected BHK-21 cells with a mixture of S di+ and S di− variants of VSV in the presence and in the absence of added DI particles. The experiments described show that DI particles can selectively alter RNA virus population dynamics and thereby influence viral evolution.

The virus referred to herein as wild-type with respect to interference phenotype (S di+) is the tsG31 of the Glasgow strain of VSV (Pringle, 1970). In 1973, this virus was used in conjunction...
Short communication

Table 1. Reconstruction experiments demonstrating selection exerted by DI particles during undiluted lytic passage and persistent infections with VSV

<table>
<thead>
<tr>
<th>Virus input (passage 1)</th>
<th>DI particle input* (passage 1)</th>
<th>Titre (p.f.u./ml)</th>
<th>Persistent infection established</th>
<th>Fraction of plaque isolates exhibiting S di- phenotype†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Passage 1</td>
<td>Passage 2</td>
<td>Passage 3</td>
</tr>
<tr>
<td>*tsG31 (S di+)</td>
<td>-</td>
<td>2.0 × 10⁹</td>
<td>3.2 × 10⁸</td>
<td>5.2 × 10⁵</td>
</tr>
<tr>
<td>75-day SP (S di-)</td>
<td>-</td>
<td>1.2 × 10⁹</td>
<td>2.8 × 10⁷</td>
<td>8.0 × 10⁷</td>
</tr>
<tr>
<td>tsG31</td>
<td>tsG31</td>
<td>1.10 × 10⁷</td>
<td>1.9 × 10⁶</td>
<td>9.0 × 10⁴</td>
</tr>
<tr>
<td>75-day SP</td>
<td>tsG31</td>
<td>6.3 × 10⁷</td>
<td>2.5 × 10⁷</td>
<td>3.3 × 10⁵</td>
</tr>
<tr>
<td>75-day SP</td>
<td>tsG31</td>
<td>9.3 × 10⁸</td>
<td>3.4 × 10⁸</td>
<td>1.4 × 10⁵</td>
</tr>
<tr>
<td>tsG31 + 75-day SP</td>
<td>-</td>
<td>1.9 × 10⁹</td>
<td>3.6 × 10⁶</td>
<td>4.0 × 10⁵</td>
</tr>
<tr>
<td>tsG31 + 75-day SP</td>
<td>tsG31</td>
<td>1.3 × 10⁷</td>
<td>9.4 × 10⁵</td>
<td>4.7 × 10⁷</td>
</tr>
<tr>
<td>tsG31 + 75-day SP</td>
<td>75-day SP</td>
<td>7.5 × 10⁷</td>
<td>2.8 × 10⁶</td>
<td>5.9 × 10⁵</td>
</tr>
</tbody>
</table>

* The tsG31 DI particle used was the ST2 (snapback) DI particle (Perrault & Leavitt, 1977). The 75-day DI particle was isolated by passaging in BHK-21 cells, the virus and DI particles released from the CAR4 persistent infection at 75 days (Horodyski et al., 1983).

† Plaques were isolated from the fourth undiluted lytic passage and from the persistent infection after 1 month. High titre clonal pools of these isolates were grown in BHK-21 cells. The interference phenotype was determined by co-infecting monolayers of BHK-21 cells with a high m.o.i. ( > 5 p.f.u./cell) of the virus isolate along with 3 DI units of purified tsG31 particles. The yield of DI particles and standard virus in the progeny was determined spectrophotometrically as previously described (Horodyski et al., 1983). A ratio of DI particles to standard virus of greater than 10 is indicative of strong interference (S di+ phenotype). A ratio of less than 0.1 is indicative of the mutant S di- phenotype.

The values in parentheses refer to the percentage of virus in the population with a small plaque morphology consistent with its being of S di- phenotype.

with its homologous DI particle to establish a continuous long-term persistent infection in BHK-21 cells (Holland & Villarreal, 1974). The resulting carrier cells are designated CAR4. After 75 days of persistence, we isolated from these CAR4 cells a small plaque (SP), S di- virus mutant which was approximately 90-fold less sensitive to interference mediated by the tsG31 (wild-type) DI particle (Horodyski & Holland, 1980; Horodyski et al., 1983). The DI particle which was also isolated from this persistent infection at 75 days exhibits a phenotype distinct from wild-type DI particles in that it is able to interfere effectively with the replication of both S di+ and S di- viruses described above, although more activity is always seen with the S di+ helper virus (Horodyski et al., 1983). Since the S di+ and S di- viruses exhibit large differences in interference by wild-type DI particles, it was important to determine whether the presence of DI particles in a mixed virus population alters the competitive properties of S di+ and S di- viruses during persistence or lytic passage.

BHK-21 cells were infected with either S di+ (tsG31) or S di- (75-day SP) viruses alone at an m.o.i. of 5 p.f.u./cell, or else with both viruses each added at an m.o.i. of 2.5 p.f.u./cell. In those cultures receiving DI particles, approximately 25 DI units (Bellett & Cooper, 1959) of either wild-type (tsG31) or mutant (75-day) DI particles were added at the same time as the infectious virus (see Table 1). Infections were carried out for 24 h at 33 °C. Virus from these initial infections was passaged undiluted in BHK-21 cells adsorbing the virus present in 1 ml of the culture fluid from the previous passage. Undiluted passage ensures the presence of large amounts of DI particles in the population (Cooper & Bellett, 1959). We also established persistent infections by adding fresh medium to the surviving cells of the initial infections.

Virus yields determined by plaque assay are shown in Table 1. Strong interference with virus replication ( > 99%) was seen in control experiments in which wild-type DI particles co-infected
with S\textsuperscript{di+} virus, whereas only slight (22\%) interference was seen when an identical amount of wild-type DI particles co-infected with S\textsuperscript{di-} virus. The mutant DI particle interfered strongly (>95\%) with both S\textsuperscript{di+} and S\textsuperscript{di-} virus at the level of DI particles used in this experiment, although slightly more interfering activity was always observed when S\textsuperscript{di+} virus was used.

In cultures receiving both S\textsuperscript{di+} and S\textsuperscript{di-} virus, the S\textsuperscript{di+} virus is easily identified by its large plaque phenotype. The percentage of small plaque virus in the population is given by the values in parentheses in Table 1. No strong selection of either virus was evident at the first passage in the presence or absence of either DI particle. By the second undiluted passage, selection of S\textsuperscript{di-} virus by the wild-type DI particle and selection of S\textsuperscript{di+} virus in the presence of the mutant DI particle was detectable. This selection was strongly evident by the fourth undiluted passage in which over 95\% of the viruses present are of the small plaque phenotype when wild-type DI particles were present in the initial infection. An equally strong selection of the wild-type large plaque phenotype was seen when the mutant DI particles were present. No strong selective advantage of either virus type was seen by the fourth passage when DI particles were not added to the initial infection.

To confirm that the plaque size phenotype assayed above generally correlated with the interference phenotype of the S\textsuperscript{di+} and S\textsuperscript{di-} virus, six plaques were picked at terminal dilution from the supernatant of the fourth undiluted passage from the mixed virus infections containing the wild-type DI particle, and six plaques were also picked from those containing mutant 75-day DI particles. High titre pools were prepared from these isolates and their interference phenotype was confirmed by co-infecting BHK-21 cells with a high m.o.i. (>2 p.f.u./cell) of the virus isolate and 3 DI units of the wild-type DI particles. Table 1 shows that all six small plaque isolates derived from the fourth undiluted passage were of the S\textsuperscript{di-} mutant interference phenotype when the wild-type DI particle was present in the initial infections. Similarly, all six isolates were of the S\textsuperscript{di+} phenotype when the mutant 75-day SP particle was present in the initial infection. Therefore, the small plaque size regularly correlated with the S\textsuperscript{di-} mutant phenotype in these populations. We conclude that DI particles can profoundly alter the population equilibrium advantage of otherwise dominant virus variants (Domingo\textit{ et al.}, 1978; Holland\textit{ et al.}, 1982).

We also determined whether the selection effects exerted by DI particles described above for undiluted lytic passage are also seen in persistent infections of BHK-21 cells. Table 1 demonstrates that persistent infections became established only when DI particles greatly reduced virus replication, and therefore a persistent infection could not be established when S\textsuperscript{di-} virus co-infected with wild-type DI particles at the levels used in this experiment.

Plaques were isolated after 1 month from both of the persistent infections established by mixed virus inocula. The interference phenotype of these isolates was determined as described above, and the results are shown in Table 1. All ten plaques isolated from the persistent infection established in the presence of the wild-type DI particle were of the S\textsuperscript{di-} phenotype. When the persistent infection was established using the mutant DI particle, seven out of nine plaques tested exhibited an S\textsuperscript{di+} interference phenotype. Therefore, the same selective effects mediated by DI particles in lytic undiluted virus passage were also observed during persistent infection.

When an S\textsuperscript{di-} mutant initially appears during persistent infection or undiluted lytic passage initiated with cloned wild-type (S\textsuperscript{di+}) virus and DI particles, it is originally present in very low ratios compared to the original S\textsuperscript{di+} virus. We therefore determined whether the selection of S\textsuperscript{di-} virus by wild-type DI particles also occurs even when the S\textsuperscript{di+} virus is initially present in vast excess. We infected BHK-21 cells with a high m.o.i. (2-5 p.f.u./cell) of wild-type S\textsuperscript{di+} virus, plus S\textsuperscript{di-} mutant virus at a range of multiplicities from 0-00025 p.f.u./cell to 2-5 p.f.u./cell, and in each case co-infect ed with 25 DI units of wild-type DI particles added at the same time. Virus was passaged undiluted in BHK-21 cells as described above and plaques were picked at various passage numbers from each passage series and the interference phenotype of these isolates was determined as described in the footnote to Table 1. Table 2 shows that the selection of S\textsuperscript{di-} mutant virus occurred even at low initial levels of S\textsuperscript{di-} virus. When the S\textsuperscript{di-} virus was originally present at a level of 1\% of the total population, a selection by wild-type DI particles occurred and the S\textsuperscript{di-} mutant constituted the majority of the population following ten un-
The above results show that the presence of DI particles can affect virus evolution. DI particles can exert continuing selective pressures, even upon S di− mutants, because new DI particles are generated which interfere with S di− parental virus mutants until they once again mutate to escape these more recent DI particles, etc. It is not yet clear whether these rounds of interference-escape-interference-escape..., can proceed indefinitely, but at least three such rounds of selection have been observed so far (Horodyski et al., 1983).

There was no selection of either virus type during the first passage when both S di+ and S di− viruses co-infected together in the presence of wild-type DI particles (Table 1). However, the observed interference with both virus types could be attributed to replications of wild-type DI particles by the S di+ helper virus. Very high ratios of wild-type DI particles can interfere with S di− virus mutants (Horodyski et al., 1983).

Obviously, many other factors besides the presence of DI particles contribute to the rapid accumulation of mutants seen during persistent infection or high multiplicity passages. These factors include generation of temperature-sensitive mutants (Youngner et al., 1978), the presence of interferon (Ramseur & Friedman, 1977; Nishiyama et al., 1978), generation of virus mutants that can interfere with the growth of wild-type virus (Spindler & Holland, 1982) and selection of mutants with altered cytopathology (Holland et al., 1979; Spindler et al., 1982) and those with an altered RNA synthesis phenotype (Frey & Youngner, 1982). It is clear, however, that when DI particles are present, they are important factors able to upset the population equilibrium dominance of otherwise dominant virus variants, thereby leading to the continuing selection of new mutant genotypes.

We thank Dr Stuart Nichol for many helpful discussions and Estelle Bussey for excellent technical assistance. This work was supported by NIH grant number AI 14627.

REFERENCES


Short communication


(Received 12 October 1983)