SUMMARY

Multiply cloned variants of vesicular stomatitis virus (VSV) were found to generate/amplify defective interfering (DI) particles at a rate greatly exceeding the rates normally observed for wild-type VSV (or for other mutants of VSV). A single undiluted passage of the first clonal pool of this variant virus produced concentrated visible bands of DI particles on sucrose gradients whereas wild-type and other mutant strains of VSV required from three to six or more serial undiluted passages. Since DI particle amplification by wild-type VSV at each undiluted passage can exceed 10 000-fold enrichment, these variant virus clones were generating/amplifying DI particles many millions of times more rapidly than were wild-type and other mutant strains of VSV. This rate of generation/amplification is so high that it was not feasible to obtain accurate estimates of the rates of generation (or amplification) of these DI particles.

Stampfer et al. (1971) first demonstrated that defective interfering (DI) particles can be eliminated from vesicular stomatitis virus (VSV) stocks by cloning, preferably multiple cloning, of infectious virus. Holland et al. (1976a) demonstrated that even the first clonal pools prepared from multiply cloned virus are already contaminated with seed quantities of newly generated DI particles, and this was confirmed for both VSV and Sendai virus DI particles (Kolakofsky, 1979, 1982), for influenza virus DI particles (Janda et al., 1979) and even for simian virus 40 DI particles (Norkin & Tirrell, 1982). Because high multiplicity co-infection of cells with helper virus allows reproducible estimations of the amplification factor of varying amounts of added DI particles (Holland & Villarreal, 1975), it is possible to estimate the rate at which viable competitive DI particles are generated by pure clonal stocks of VSV to be about $10^{-7}$ to $10^{-8}$ DI particles generated per replication of wild-type helper virus (Holland et al., 1976b). DI particle generation rates have not been accurately estimated for other viruses, but they are probably of the same order of magnitude as for VSV in the case of Sendai virus (Kolakofsky, 1979) and influenza viruses (Janda et al., 1979), based upon the number of serial passages required to detect DI particles. However, this might vary considerably with cell type, virus strain, virus passage history, etc. We show here that certain VSV variants isolated following many years of persistent infection of BHK-21 cells in vitro generate/amplify DI particles at rates greatly exceeding those of wild-type VSV and of other virus mutants. Any significant replication of these VSV mutants must be accompanied by DI particle generation and amplification, and once amplified they interfere strongly with parental helper virus.

These variants were isolated after 110 and 120 months, respectively, of the DI particle-mediated persistent VSV infection of BHK-21 cells designated ‘CAR 4’ (Holland & Villarreal, 1974). Virus isolates from this carrier cell line show a high mutation frequency and rapid evolution in all proteins and non-coding terminal regions (Spindler et al., 1982; Rowlands et al., 1980; for review, see Holland et al., 1982). Many mutant phenotypes of VSV have been isolated from CAR 4 carrier cells, including slow-growing viruses with extremely high particle-to-infectivity ratios as described by Spindler et al. (1982). This phenotype was unstable, as after several low m.o.i. passages the particle-to-infectivity ratio fell (as infectious virus yields
increased) to near wild-type levels. We isolated numerous slow-growing avirulent virus phenotypes at later times during this prolonged persistent infection, and at some points faster growing phenotypes were also observed. At 110 months we isolated mutant virus with a new and quite stable phenotype (herein designated 110 month CAR 4 virus). Even after subjecting this mutant to repeated plaque-to-plaque passages, it proved impossible to prepare a high titre virus pool of 110 month CAR 4 which was not heavily contaminated with DI particles. The first passage pool (prepared by infection at very low m.o.i. necessarily, because input virus was derived from a plaque) was always of very low titre (below 10⁶ p.f.u./ml) and the second passage, despite an input m.o.i. of less than 0-1, was always heavily contaminated with DI particles. Subsequent passages exhibit greatly reduced yields of helper virus and of DI particles (autointerference).

This was unprecedented behaviour for any multiply cloned VSV mutant from CAR 4 (Spindler et al., 1982) or from any other VSV source employed in this laboratory. To confirm and to characterize this phenotype further, we prepared successively cloned plaques of this 110 month CAR 4 isolate along with six other VSV isolates for comparative characterization. These included an earlier CAR 4 virus isolated after 60 months of persistent infection (60 month CAR 4) and later CAR 4 isolates (120 month CAR 4, 124 month CAR 4 and 136 month CAR 4). As a control for the effects of the slow growth rate, we also characterized multiply cloned plaque isolates of some slow growing VSV mutants from a series of undiluted lytic passages (Spindler et al., 1982) from passage 223 (p223), p257 and p287. Finally, we characterized tsG31 (Pringle, 1970), the original virus for both of these mutant sources. All preparations and assays were done with BHK-21 cells maintained with Eagle's MEM plus 7% heat-inactivated calf serum. All infections were at 33 °C for these temperature-sensitive viruses. All viruses examined here were successively cloned at least four times; final working virus was picked from well isolated plaques, and diluted with 1 ml of medium (titres ranged from 5 × 10³ to 10⁵ p.f.u./ml). They were stored frozen at −70 °C until used to initiate parallel undiluted passage series.

Each of the above multiply cloned plaque isolates was passaged undiluted in triplicate passage series starting with adsorption of 0-1 ml of the picked plaque to each of three monolayers of about 3 × 10⁷ BHK-21 cells. When viral cytopathology was complete (usually 1 to 3 days depending upon virus growth rates), 1-0 ml (of 28 ml total) virus-containing medium was used to infect a fresh monolayer of about 3 × 10⁷ cells for each of the triplicate passage series. These serial undiluted passages were carried out successively until DI particles appeared during sucrose gradient analyses of the remainder of virus from each yield. Table 1 shows the results of these analyses. In every case the DI particles first produced visible bands in sucrose gradients at the same passage level in the three parallel passage series (for each specific VSV isolate). However, as can be seen in Table 1, most of the clonal isolates of VSV mutants produced visible DI particle bands between the third and the fifth undiluted passage, but strikingly the 110 month and 120 month CAR 4 mutants consistently (in all three of the triplicate passage series) produced large amounts of DI particles during first passage from a clonal pool. Kolakofsky (1982) also consistently observed wild-type VSV DI particle genome RNAs becoming visible on gels at passage four from a DI particle-free plaque isolate. Each of the above parallel passage series for 110 month CAR 4 virus and 120 month CAR 4 virus produced DI particles of different sizes than were produced in the other two passages. This assay for randomness of generation (Holland et al., 1976a) demonstrates that the original plaque-picked low titre pools of 110 month and 120 month virus did not contain seed quantities of DI particles and therefore that the observed DI particles were newly generated during the first undiluted passage. These DI particles observed during the first passage from clonal pools were strongly interfering because second passages showed strong autointerference (greatly reduced yields of virus and of DI particles).

We determined that this unusual phenotype of rapid DI particle generation was the predominant mutant phenotype in the CAR 4 carrier cells at 110 and 120 months, by picking three different plaques from the former and two from the latter carrier cells after co-cultivation with BHK-21 cells. All five plaques exhibited this rapid generation phenotype. Note in Table 1, however, that this phenotype had disappeared as a predominant mutant type by 124 months and
Table 1. Appearance of visible DI particle bands on sucrose gradients following successive undiluted passages of different VSV clonal pools

<table>
<thead>
<tr>
<th>Virus tested</th>
<th>DI particle appearance* at passage number†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>One</td>
</tr>
<tr>
<td>tsG3</td>
<td></td>
</tr>
<tr>
<td>60 Month (CAR 4)‡</td>
<td>−</td>
</tr>
<tr>
<td>110 Month (CAR 4)</td>
<td></td>
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<tr>
<td>120 Month (CAR 4)</td>
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<tr>
<td>124 Month (CAR 4)</td>
<td>−</td>
</tr>
<tr>
<td>136 Month (CAR 4)</td>
<td>−</td>
</tr>
<tr>
<td>p223 (u.p.)§</td>
<td>−</td>
</tr>
<tr>
<td>p257 (u.p.)</td>
<td></td>
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<tr>
<td>p287 (u.p.)</td>
<td>−</td>
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</table>

* DI particle bands were barely visible (±) at concentrations of less than 1% of viral yield as determined by spectrophotometric analysis (Horodyski et al., 1983). A scoring of (+) indicates that DI particles were >5% of total viral particle yield. All tests were done in triplicate.
† The passage number begins with passage 1 from one of three separate clonal pools as, in all cases, independent passages were done in triplicate and the randomness of generation of DI particles was verified to rule out contamination of original viral plaques by seed quantities of DI particles.
‡ CAR 4, Persistently infected carrier line of BHK-21 cells (see text).
§ u.p., Undiluted passage series (see text).

134 months of persistent VSV infection in CAR 4 cells. It is not clear why this phenotype disappeared after being dominant for at least 10 months, but RNA virus populations can evolve very rapidly during persistence (Holland et al., 1982).

To examine the stability of this rapid generation phenotype, a clonal pool of 110 month CAR 4 was passaged five times at low multiplicity (0.1 ml of 1/100 dilution) and then successively recloned three times and analysed as before. This virus now replicated to 100-fold higher titres (about $5 \times 10^8$ p.f.u./ml) in a manner analogous to the behaviour of 70 month CAR 4 mutant virus (Spindler et al., 1982). However, random-sized DI particles were still always seen on the first high multiplicity passages of a clonal pool, showing that the rapid DI generation/amplification phenotype was rather stably maintained.

It would be interesting to determine the approximate rates of generation and amplification of DI particles by these VSV variants but this is not now feasible, because determination of the amplification factor per synchronous viral growth cycle requires the availability of high m.o.i. helper virus inputs which will not generate detectable DI particle bands (Holland & Villarreal, 1975), and the high rates of DI particle generation/amplification observed above preclude this. Similarly, determination of DI particle generation rates requires knowledge of approximate rates of amplification per cell replication cycle (Holland et al., 1976b).

In summary, the above results show that these VSV variants generate/amplify DI particles so rapidly that significant virus replication must nearly always be accompanied by DI particle replication and interference in these BHK-21 cells. This could have exerted a considerable attenuating effect upon virus virulence for host cells at the times when these variants were present during persistent infection. It is likely that similar variants might arise frequently in nature during acute (as well as persistent) infections. In vitro virus assay systems tend naturally to select for rapidly replicating, larger plaque types of virus variant, whereas variants which generate/amplify DI particles at high rates are inherently more difficult to isolate and to characterize. These should probably be less virulent, but we have not yet determined the LD_{50} of these mutants in mice. Presumably the rate of generation/amplification of DI particles would be regulated by viral replication/encapsidation genes (Leppert et al., 1977; Huang, 1977) and mutants of viral complementation group I (L protein polymerase gene) are the most abundant mutants of VSV (Pringle, 1970, 1977; Szilagyi & Pringle, 1979). Some RNA viruses might generate DI particles extremely rapidly even from common wild-type and mutant strains. For example, rabies virus (Crick & Brown, 1974; Holland et al., 1976a; Kawai & Matsumoto, 1982).
often generates DI particles rapidly, as does lymphocytic choriomeningitis virus (Popescu et al., 1976) and Semliki Forest virus (Barrett et al., 1981). In contrast, picornaviruses generate/amplify DI particles at very low rates (Cole & Baltimore, 1973; McClure et al., 1980; Radloff & Young, 1983).

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REFERENCES


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