The role of environmental factors on the evolution of phenotypic diversity in vesicular stomatitis virus populations

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INTRODUCTION

Virus emergence and re-emergence, drug resistance, antibody escape and changes in host tropism are all important evolutionary outcomes that depend on the availability of appropriate genetic variation. RNA viruses are superbly equipped to produce diversity due to their high mutation rates and the potential, in some cases, to exchange genetic information. RNA-dependent RNA polymerases and reverse transcriptases are error prone (Drake & Holland, 1999; Sanjuan et al., 2010) and, in the absence of correcting mechanisms (Steinhauer et al., 1992), riboviruses incorporate an average of one mutation each time that a full genome is copied (Domingo & Holland, 1997). Recombination and reassortment increase the ability to explore sequence space (Domingo et al., 2012).

Virus survival depends on the availability of mutants when the selective environment changes. However, the competitive exclusion principle states that, in the absence of niche differentiation, co-existence among variants is not possible (Gause, 2003), because the fittest genome will outcompete all other mutants. Thus, it is important to understand how genotypic and phenotypic variation is maintained in virus populations. Many organisms use phenotypic plasticity, which includes physiological and behavioural responses, to adjust to environmental changes (Badyaev, 2009). Phenotypic plasticity is usually obtained through differential gene regulation. However, RNA viruses use gene regulation to a minimal extent, if at all, perhaps because of their very small and compact genomes.

Many parameters are likely to determine the extent of diversity, including effective population size, the strength of selection, migration and the structure of the environment. Within-host bottlenecks promote diversity by allowing the survival of low-fitness components from the population. For example, infected plants can support high diversity within the host because there are severe bottlenecks during the spread of the virus and different variants colonize different branches (Ali & Roossinck, 2010; Jridi et al., 2006). Similarly, analysis of individual organs shows different variants of human immunodeficiency virus type 1 (Delassus et al., 1992; Frost et al., 2001) and West Nile virus (WNV) in different parts of the host body (Ciota...
et al., 2012a). In contrast, bottlenecks during transmission between hosts may result in the elimination of diversity during the early period of infection (Duarte et al., 1994b; Li & Roossinck, 2004).

In homogeneous environments, and under selection, there are four processes that contribute to maintaining phenotypic and genotypic diversity: heterosis dominance, negative frequency-dependent selection, recurrent beneficial mutation and recurrent deleterious mutation (Rainey et al., 2000). Heterosis dominance does not operate in the RNA viruses we are interested in here, because they are haploid. There are a few examples of negative frequency-dependent selection (Elena et al., 1997), but it is hard to assess its contribution to maintaining variation. Recurrent mutations, both beneficial and deleterious, must be important given the high mutation rates of these pathogens, and the prediction is that diversity will decrease with the fixation of beneficial mutations and increase with the generation of new mutations (Kassen & Rainey, 2004).

In heterogeneous environments, the existence of trade-offs or other costs are likely to play a role. Temporal changes in the environment correspond to seasonality (for instance, changes in the abundance of a nutrient) or to host switches in multihost infections. There is evidence of increased variation due to seasonality in bacteria but only if trade-offs occur (Rainey et al., 2000). However, in Chikungunya virus, temporal heterogeneity supplied by different cell types results in decreased phenotypic diversity as measured by drug-resistant and antibody-escape mutant frequency (Coffey & Vignuzzi, 2011). Alternation of vesicular stomatitis virus (VSV) between different cell types limits among-population diversity in virus production (Turner et al., 2010). Studies of WNV and St Louis encephalitis virus (SLEV) evolution have shown that host alternation did not limit (or promote) genotypic diversity (Jerzak et al., 2008). Kassen (2002) reviewed the literature and found no consistent pattern in the diversity changes during microbial adaptation to temporal environmental changes. Subsequent work has shown that temporal environmental variation does not necessarily support higher diversity in bacteria (Jasmin & Kassen, 2007).

Structural variation corresponds to environments with discrete patches that exert selection differently. Examples are the different cell types or organs within an infected host or the different host types in an ecosystem. Niche differentiation provides an escape from the competitive exclusion principle and should support higher diversity because each niche within the environment will select for a different specialist, and all specialists can then co-exist. Among bacteria, there are examples for (Rainey & Travisano, 1998) and against (Saxer et al., 2009) this hypothesis. There are some reports available that address adaptation to spatially heterogeneous environments (for instance, Cuevas et al., 2003), but the topic of viral diversity maintenance under these conditions is understudied.

We sought to examine the relevance of several environmental parameters on the maintenance of phenotypic diversity in VSV populations. We determined fitness distributions of strains with diverse evolutionary histories and under different conditions. We concluded that the two parameters that had the highest effect on diversity were the cell type in which evolution took place and whether replication occurred during acute or persistent infection. The latter can be explained because of consistent co-infection, which promotes the survival of deleterious mutants due to complementation (Ciota et al., 2012b; Novella et al., 2004; Wilke & Novella, 2003; Wilke et al., 2004).

**RESULTS AND DISCUSSION**

**Clonal analysis versus analysis of small samples**

Under ideal circumstances, our analyses would have consisted of determining the fitness of clones (individual plaques) picked randomly from each population (Novella et al., 2010). However, some of the strains, including those with a history of persistent infection in LL-5 cells or a history of replication in HeLa cells, included variable numbers of tiny, pinprick plaques. Because random plaque picking requires that the plaques are visible to the naked eye before staining, we felt that clonal analyses would bias our sampling of these populations substantially (Novella et al., 2007). In addition, we had established previously that, even with fairly large populations (~10^5 p.f.u., 100-fold larger than the sample size chosen for this work), it was possible to obtain a fitness distribution that reflects variation within the population (Duarte et al., 1994a). It is important to note that these populations are not expected to be homogeneous but are expected to be small enough to allow variation in sampling. Thus, we chose to measure fitness of relatively small populations (2000 p.f.u.) instead of individual clones. The disadvantage of our approach was the loss of sensitivity, but this problem was compensated for by the assurance that any differences in phenotypic variance observed during these studies were meaningful and not the result of sampling bias. The result of the study is summarized in Fig. 1, where we have shown mean CV values with the 95% CI for all strains analysed (described in Table 1).

**Diversity depends on the cell type used for selection**

Host-cell type is an obvious candidate to shape the amount of diversity within a viral population. We tested the effect of selective cell type on diversity by grouping the strains based on the cell type in which they had replicated. This criterion produced four groups of strains (Fig. 2). Strains with a history of replication in baby hamster kidney (BHK-21) cells included K25a, K25b, K80a, K80b, K80c and K80d; strains with a history of replication in LL-5 cells
included Lac80b, Lac 80d, Lper25 and Lper16; strains with a history of replication in HeLa cells included H25a, H25c; and strains with a history of replication in MDCK cells included M25a, M25c. We excluded from the analysis strains that replicated in two different cell types to avoid including the same data points in more than one group. Our results indicated that LL-5, MDCK and HeLa cells supported more diversity than BHK cells ($P<0.007$ for each pair), but there were no significant differences among these last three cell types ($P>0.15$ for each pair) (Fig. 2). In this case, low viral diversity may be a reflection of low host diversity, at least in some cases. We do not know the exact history of European HeLa and MDCKs, but the BHK-21 cells were cloned a few years ago, and the clone used in this study was selected based on its ability to produce high viral titres. In contrast, LL-5 cells are not a single cell type but a mixture of epithelioid and fibroblastoid cells of diverse shapes and sizes (Tesh & Modi, 1983).

Our results are consistent with studies on the evolution of viral genetic diversity in different hosts assuming that, generally speaking, phenotypic and genotypic variance tend to correlate. Among animal viruses, previous studies have shown that mosquitoes and mosquito cells support higher genotypic diversity of WNV than chickens (Ciota et al., 2007; Jerzak et al., 2007). In contrast, the same hosts support similar low-level nucleotide diversity of SLEV (Ciota et al., 2009). Interestingly, the same analysis applied to the predicted amino acid changes revealed increased diversity in mosquito-adapted strains (Ciota et al., 2009). Among plant viruses, Schneider & Roossinck (2001) found that host type was a key determinant of genotypic diversity for tobacco mosaic virus and cucumber mosaic virus (CMV). In both cases, pepper plants supported high genotypic diversity, whilst *Nicotiana benthamiana* tended to support low diversity, and other species such as tomato and squash supported similar diversity for CMV.

Based on the present study and existing literature, the emerging pattern is that host type is a possible determinant of the extent of phenotypic variation, but there does not appear to be a general rule.

**Fig. 1.** Phenotypic diversity, measured as fitness’ coefficient of variation (CV), for the strains studied in this article. Horizontal dashes represent the CV and vertical lines indicate 95% confidence intervals (CI). Labels for each viral strain are the same as in Table 1. Strain labels alone correspond to determinations in BHK-21 cells, labels ending in ‘inM’ correspond to determinations in Madin–Darby canine kidney (MDCK) cells and labels ending in ‘inH’ correspond to determinations in HeLa cells.

**Table 1.** Strains under investigation in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host cells</th>
<th>No. passages/cycles</th>
<th>Infection type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt/MARM U</td>
<td>BHK-21</td>
<td>0</td>
<td>NA</td>
<td>Holland et al. (1991)</td>
</tr>
<tr>
<td>K25a, K25b</td>
<td>BHK-21</td>
<td>25</td>
<td>Acute</td>
<td>Novella et al. (1999a)</td>
</tr>
<tr>
<td>K80a, K80b, K80c, K80d</td>
<td>BHK-21</td>
<td>80</td>
<td>Acute</td>
<td>Novella et al. (1999a)</td>
</tr>
<tr>
<td>Lac80b, Lac80d</td>
<td>LL-5</td>
<td>80</td>
<td>Acute</td>
<td>Novella et al. (1999a)</td>
</tr>
<tr>
<td>KLac80c</td>
<td>BHK-21/LL-5</td>
<td>160/80</td>
<td>Acute/persistent</td>
<td>Novella et al. (1999a)</td>
</tr>
<tr>
<td>KLper25b</td>
<td>BHK-21/LL-5</td>
<td>50/25</td>
<td>Persistent</td>
<td>Novella et al. (2004)</td>
</tr>
<tr>
<td>Lper16</td>
<td>LL-5</td>
<td>16</td>
<td>Persistent</td>
<td>Novella et al. (2004)</td>
</tr>
<tr>
<td>Lper25</td>
<td>LL-5</td>
<td>25</td>
<td>Acute</td>
<td>Smith-Tsurkan et al. (2010)</td>
</tr>
<tr>
<td>M25a, M25c</td>
<td>MDCK</td>
<td>25</td>
<td>Acute</td>
<td>Smith-Tsurkan et al. (2010)</td>
</tr>
<tr>
<td>MH12.5a, MH12.5c</td>
<td>MDCK/HeLa</td>
<td>25/12.5</td>
<td>Acute</td>
<td>Smith-Tsurkan et al. (2010)</td>
</tr>
<tr>
<td>MH25a, MH25c</td>
<td>MDCK/HeLa</td>
<td>50/25</td>
<td>Acute</td>
<td>Smith-Tsurkan et al. (2010)</td>
</tr>
</tbody>
</table>
Diversity may change significantly over time

In a constant environment, phenotypic variance is expected to decrease over time. The rationale is that selection should purge all except the fittest variants, leading to a loss of diversity once the best genotype is fixed. To test this prediction, we used two time series from populations evolving in a homogeneous environment, BHK-21 cells: wild type (wt)–K25a–K80a (termed Ka), and wt–K25B–K80B (termed Kb). Fig. 3 shows the changes in CV over time. For both Ka and Kb, there was an initial loss of diversity followed by a recovery to initial levels. However, diversity changes at the intermediate time point were only significant for Ka ($P = 0.0071$) and not for Kb ($P = 0.16$). This result was not completely unexpected and is reminiscent of the ‘sawtooth’ pattern found in bacterial populations, which represent periods of diversity loss during fixation of beneficial alleles, followed by periods of increased diversity brought about by mutation (Atwood et al., 1951; Notley-McRobb & Ferenci, 2000). Previous work supports this interpretation for the initial loss of phenotypic variance. Indeed, adaptation during the first ~20 passages corresponds to the fixation of beneficial variations already found within the population (Dutta et al., 2008). The recovery of diversity at passage 80 is harder to explain, because at this time the populations have reached their fitness peaks (Novella et al., 1999b) and presumably all additional beneficial variation has been fixed, so one would expect low diversity. We had two other time series in our dataset: MARM U–MH12.5a–MH25a (termed MHa) and MARM U–MH12.5c–MH25c (termed MHc) (Fig. 3). These populations were evolved in heterogeneous environments (alternating between MDCK and HeLa cells) and the differences in CV were not statistically significant ($P > 0.6$). As discussed above, differences in host-cell types (BHK-21 vs HeLa/MDCK; Fig. 2) may be contributing to the maintenance of diversity in these populations.

Most of the available literature on the evolution of viral diversity over time presents changes in genotypic, not phenotypic, diversity. The extent of diversity clearly depends on individual viral species. WNV and SLEV are flaviviruses with very similar natural cycles, which, like VSV, include alternation between arthropods (in this case, mosquitoes) and vertebrates (Ciota & Kramer, 2010). In experiments starting with a homogeneous progenitor produced from cDNA, WNV passaged in mosquitoes or mosquito cells showed a significant increase in genotypic diversity (Ciota et al., 2007; Jerzak et al., 2007). Compared with WNV, SLEV populations tended to remain relatively homogeneous at the nucleotide level in both mosquitoes and chickens, but there seemed to be a tendency for increased diversity with time (Ciota et al., 2008). Plant viruses behave in a similar manner in that genotypic diversity is a function of the viral species. Specific host–virus pairs produce predictable levels of diversity, but the level of diversity does not change over time (Schneider & Roossinck, 2000).

Temporally heterogeneous environments do not seem to support higher diversity

For this analysis, we divided the strains into two groups based on the complexity of the selective environment (Fig. 4). Strains that had a history of replication in a homogeneous environment (single cell type) comprised K25a, K25b, K80a, K80b, K80c, K80d, Lac80b, Lac80d, Lper25, Lper16, H25a, H25c, M25a and M25c, whilst strains that had a history of replication in temporally heterogeneous environments (alternating between two cell types) comprised KLac80c, KLper25b, MH12.5a, MH12.5c, MH25a and MH25c. All data show measurements of CV on BHK-21 cells. Our determinations of diversity produced
a CV of 0.59 for homogeneous environments and 0.53 for heterogeneous environments, and this small difference was not statistically significant ($P>0.15$).

Theoretically, temporally heterogeneous environments were expected to support somewhat higher overall diversity than homogeneous environments, both phenotypically and genotypically. However, previous work in several systems, including viruses and bacteria, has shown little evidence in support of this prediction (Kassen, 2002). Furthermore, Chikungunya virus (CHIKV) that alternates between insect and mammalian cells tends to have lower phenotypic diversity than CHIKV that replicates in a single cell type (Coffey & Vignuzzi, 2011). Consistent with this result, analysis of VSV phenotypic diversity after adapting to homogeneous (MDCK or HeLa cells) or temporally heterogeneous (alternating between MDCK and HeLa cells) environments showed lower phenotypic diversity in strains with a history of alternation than in strains adapted to single cell types (Turner et al., 2010). In this case, diversity was measured as among-population variance of viral titres in the cell type where selection took place and in novel cell types. It is important to note that we may have missed differences in diversity due to limits in the sensitivity of our method: we analysed samples of 2000 p.f.u. instead of clones. However, this is one particular case where clonal analysis would have been unwise due to the presence of pinprick plaques in some of the strains.

Other reports have presented data on changes in genotypic diversity. The diversity of WNV alternating between mosquitoes and chickens was similar to that of WNV replicating only in mosquitoes and was higher than the diversity of WNV replicating in chickens (Jerzak et al., 2008). Similar studies were not performed for SLEV, but the low levels of diversity shown by each of the host types (Ciota et al., 2008, 2009) and the fact that natural populations from Texas and California are losing diversity (Ciota et al., 2011) are inconsistent with increased diversity during alternation. Overall, we were unable to find a correlation between temporal environmental heterogeneity and diversity.

**Generalists and specialists harbour similar amounts of diversity**

Ecological theory predicts that strains adapting to homogeneous environments will become specialists with the ability to replicate well in the selective host but not in alternative hosts (Buckling et al., 2003). In contrast, strains adapting to heterogeneous environments will become generalists, with the ability to replicate to some extent in multiple hosts but less well than the specialist in any given host (Whitlock, 1992). However, our work and that of others is inconsistent with this assumption, because homogeneous environments frequently select for generalists and heterogeneous environments may select for a specialist (Novella et al., 2011). Therefore, we considered the possibility that the relevant parameter was the strain’s niche breadth (specialist vs generalist) and we divided the strains into two groups based on their ability to replicate in different hosts. The first group represented the generalists, which were the strains with high fitness across environments, and comprised K25a, K25b, K80a, K80b, K80c, K80d, Lac80b, Lac80d, M25a, M25c, KLac80c, MH12.5a, MH12.5c, MH25a and MH25c. The second group represented the specialists, which had high fitness in a selective environment but low fitness in one or more alternative environments, and comprised Lper25, Lper16, KLper25, H25a and H25c. All data corresponded to measurements of CV on BHK-21 cells (Fig. 5).

The differences in CV between specialists (0.87) and generalists (0.48) were statistically significant ($P<0.0001$). The statistical significance was due to the two high values from populations with a history of persistence, Lper25 and Lper16 (see below); without these values, the extent of diversity in specialists decreased substantially (0.43), and the statistical significance of the differences was lost ($P=0.0577$) (Fig. 5). This is perhaps a case in which our analysis may have suffered from the loss of sensitivity in the method. To our knowledge, there are no other studies looking at phenotypic variation in viral generalists and specialists independently of their history, although Whitlock (1996) proposed that the key parameter determining host breadth is the evolutionary rate: specialists have a higher rate of fixation of beneficial alleles and a lower rate of fixation of deleterious alleles.

**Persistent infections support the highest amount of diversity**

The next parameter under investigation was the strategy of replication. Viral infection can proceed as an acute infection, with rapid and relatively large virus production...
and, usually, cell death. Alternatively, it can proceed as a persistent infection, with low but continuous viral production and little or no cell death. In the case of VSV and other arboviruses, vertebrate infection is usually acute and cytolytic, whilst vector infection is persistent. These two strategies of replication represent different selective pressures (Presloid et al., 2008; Zárate & Novella, 2004). We analysed strains adapted to LL-5 cells and divided them into two groups (Fig. 6). The first group represented strains from acute infections, for which viral progeny had been recovered after the initial peak of rapid replication, and comprised Lac80b, Lac80d and KLac80c. The second group represented viruses from persistent infections, for which infected cells – instead of virus – had been passaged, and comprised Lper25 and Lper16. The amount of phenotypic diversity observed in persistent strains (mean CV = 1.28) was higher than the diversity identified in acute strains (mean CV = 0.54) and this difference was statistically significant (P = 0.0121). Furthermore, the CV for persistent strains was the highest of the entire dataset. This observation is consistent with previous work, and is probably due to the differences in m.o.i. during replication. Whilst acute infections periodically go through replication at low m.o.i. (during transmission), persistent infections presumably maintain high levels of co-infection during virus replication, which favours complementation (Novella et al., 2007). Complementation, in turn, limits the efficiency of selection and promotes the survival of deleterious mutants (Novella et al., 2004; Wilke & Novella, 2003; Wilke et al., 2004). Indeed, the periodic inclusion of a step of acute BHK-21 cell infection (KLper25b), during which low m.o.i. restored the effect of selection, resulted in one of the lowest levels of diversity in the complete dataset (CV = 0.307) (Fig. 1). The KLper25b CV value was similar to those of BHK-21-adapted strains (CVs between 0.213 and 0.320) and lower than those for Lper25 (CV = 1.379) and Lper16 (CV = 1.210) (Fig. 1). It is worth noting that the fitness evolution of Lper25 and KLper25b was virtually identical (Zárate & Novella, 2004). Both showed dramatic, and similar, fitness gains in LL-5 cells that stabilized after ~15 passages, as well as some initial fitness loss in BHK-21 cells that recovered to some extent towards the end of the experiment. Genotypically, the evolution of the two strains was also virtually identical, with mostly identical changes in the consensus sequence. However, as demonstrated here, the composition of each population was quite different. By contrast, Lper25 and Lper16 had different ancestors (wt and MARM U, respectively) and accumulated completely different mutations (Novella et al., 2007), but both strains supported similar (and very high) levels of diversity. In conclusion, persistence and high m.o.i. support high phenotypic diversity.

**Measuring diversity in different cell types does not reveal different amounts of variation**

Fitness is one of many potential phenotypes that we could have chosen to study. Other groups have performed similar analyses on other phenotypes such as antibody- or drug-sensitivity mutants (Coffey & Vignuzzi, 2011). Conceptually, fitness always represents a phenotype in a specific environment, and it was reasonable to hypothesize that the cell type used to obtain fitness distributions may have a role in the extent of diversity that we can measure. We divided the data into three groups based on the cell type in which we determined diversity (Fig. 7). For this analysis, we only included CV values for which we had data for more than one test cell type. CV values obtained in BHK cells comprised strains H25a, H25c, M25a, M25c, MH25a, H25b, M25b, MH25b, H25d, M25d, MH25d and H25e, M25e, MH25e.
lack of trade-offs observed during adaptation to these cell lines (Novella et al., 1999a; Smith-Tsurkan et al., 2010). The only case in which adaptation to acute infection of BHK-21, MDCK, HeLa or LL-5 cells resulted in fitness loss for other cell types was that represented by H25a and H25c, which suffered significant fitness losses in MDCK cells; H25c also had fitness losses in BHK-212 cells, but H25a did not (Smith-Tsurkan et al., 2010). Perhaps if we had carried out our determinations in more disparate environments, the result would have been different, but it is hard to predict what such an environment would be.

Many other parameters may affect, to different extents, the maintenance of viral diversity, including the effective population size, spatial environmental heterogeneity and migration. It is important to continue analyses of these parameters because they are key to answering very fundamental (and practical) questions, including how viruses evolve, how they emerge and how new species come to be.

**METHODS**

**Cells and viruses.** All plaque assays were carried out in BHK-21 cells from John Holland’s laboratory (University California, San Diego, CA, USA; Holland et al., 1991). We performed fitness determinations on BHK-21 cells, and also on human HeLa cells and MDCK cells from the European Collection of Cell Cultures. We used minimal essential medium (MEM) supplemented with 7% heat-inactivated, bovine calf serum (BCS) and 0.6% proteose peptone (PP3) for the growth of BHK-21 cells. For HeLa and MDCK cells, MEM supplemented with 10% FBS was used. The progenitor of all strains was the wt VSV, Indiana serotype (Mudd-Summers strain) (Holland et al., 1991). MARM U is a clone of the wt obtained in the presence of mAb II (Lefrancois & Lyles, 1982) and has a single nucleotide substitution in the G gene that translates into a G257A mutation. All strains under investigation were the result of replication under conditions of predominant positive natural selection. Cells used as selective environments to generate the strains used in this report included BHK-21, MDCK, HeLa and sandfly LL-5 cells (Tesh & Modi, 1983).

Passage regimes included either replication in homogeneous environments, for which we performed repeated transmissions on a single cell type, or replication in temporally heterogeneous environments, for which we transmitted the virus back and forth between two different cell types. Table 1 lists all the strains under investigation and shows their history. The labelling of MH strains in this report (based on cycles) was slightly different from that in our previous report (based on passages) to be consistent for all strains: MH12.5 corresponds to MH25 and MH25 corresponds to MH50 in Smith-Tsurkan et al. (2010).

**Passage conditions.** Acute passages consisted of infections at low m.o.i. (0.1 p.f.u. per cell in BHK-21 and LL-5 cells, and 0.01 p.f.u. per cell in MDCK and HeLa cells). Virus populations were allowed to replicate for 48 h (LL-5 cells) or until the cytopathic effect was complete in the rest of the cell types (24–48 h). Persistent infection of LL-5 cells was initiated at a low m.o.i. of 0.1. The infection was allowed to proceed for 2 weeks, with medium replacement on days 4 and 11. On day 14, the cells were split and a new flask was seeded with 1/20th of the recovered cells. Thus, for persistent passages, the infected cells, rather than the supernatant virus, were passaged.

**Measurement of phenotypic variation.** The phenotype under investigation was relative fitness, defined as the overall replicative
ability. To measure fitness, we used a mAb 11 resistance mutation as a genetic marker for one of the competitors. We mixed test and reference viruses and the mixture was used for two purposes: first, we used a diluted sample to carry out a competition passage in the appropriate cell type and, secondly, we plated in triplicate in the presence and absence of mAb 11 to determine the exact ratio of the two competitors (R0). After 10 min at room temperature followed by 40 min at 37°C, we added MEM + FBS to the passage and MEM + BC + 0.1% agarose with or without mAb 11 to the plaque assays, which were developed at 20–48 h post-infection (p.i.). Once cytopathic effect was complete (20–48 h p.i.), we recovered the viral progeny from the competition passages and performed a new plaque assay in the presence and absence of mAb 11 to determine the ratio after competition (R1). Fitness was defined as R1/R0.

To obtain each fitness distribution, we carried out competitions using 2000 p.f.u. test virus against the appropriate reference (wt or MARM U). For each strain, we carried out a set of 20 independent determinations. As a control, we generated a set of 20 determinations between 2000 p.f.u. wt and 2000 p.f.u. MARM U. To control for differences in fitness among strains, we normalized each fitness determination by the mean fitness of the corresponding population.

Statistical analyses. Results were expressed as normalized variance values (CV). We analysed changes in CV using the Brown and Forsythe test for homogeneity of variances (Brown & Forsythe, 1974). Significance was set at P≤0.05 for all analyses. We used Bonferroni correction to prevent the accumulation of false positives due to multiple comparisons. Statistical analyses were performed using SAS version 9.2 and R version 2.15.

ACKNOWLEDGEMENTS

We are grateful to Douglas Lyles for the gift of the 11 hybridoma and Rees Kassen for helpful comments, suggestions and discussion. This work was supported by NIH grant R01 AI065960.

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