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Emergence of Mammalian Cell-Adapted Vesicular Stomatitis Virus from Persistent Infections of Insect Vector Cells▽

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Arboviruses (arthropod-borne viruses) represent quintessential generalists, with the ability to infect and perform well in multiple hosts. However, antagonistic pleiotropy imposed a cost during the adaptation to persistent replication of vesicular stomatitis virus in sand fly cells and resulted in strains that initially replicated poorly in hamster cells, even when the virus was allowed to replicate periodically in the latter. Once a debilitated strain started replicating continuously in mammalian cells, fitness increased significantly. Fitness recovery did not entail back mutations or compensatory mutations, but instead, we observed the replacement of persistence-adapted genomes by mammalian cell-adapted strains with a full set of new, unrelated sequence changes. These mammalian cell-adapted genomes were present at low frequencies in the populations with a history of persistence for up to a year and quickly became dominant during mammalian infection, but coexistence was not stable in the long term. Periodic acute replication in mammalian cells likely contributed to extending the survival of minority genomes, but these genomes were also found in strictly persistent populations.

The molecular mechanisms underlying adaptation are one of the most crucial topics of evolutionary genetics. In the case of viruses, this question is also important because adaptation will have an impact on transmission and on the pathogenesis caused by infection and, thus, on our ability to control viral diseases (6). Adaptation of a viral population to a novel environment occurs when variants with higher fitness become dominant in the population. The source of those variants depends on the evolutionary history of the strain. Viruses with a history of bottlenecks usually have low fitness, and their recovery takes place because of beneficial variation during replication in the form of reversion or compensation, resulting in the accumulation of mutations in those unfit genomes (9, 19). In viruses that undergo homologous recombination, adaptation may be further favored because recombination may bring two or more beneficial mutations together in a single genome (3). A second mechanism that results in viruses that are unfit in a particular environment is trade-off, when adaptation to one environment results in maladaptation to a second environment. There are two situations that exemplify maladaptation due to trade-offs. First, arbovirus adaptation to insect cells sometimes results in populations that have low fitness in mammalian cells (5, 10, 33). Second, antibody escape and drug-resistant mutants usually have low fitness in the absence of the selective pressure (18, 20, 22, 25). We have used vesicular stomatitis virus (VSV) to study how low-fitness viral populations recover when maladaptation is the result of trade-offs.

VSV is the prototype of the *Rhabdoviridae* family (24). Its genome is a molecule of single-stranded, negative-sense RNA that codes for at least five proteins. The nucleoprotein (N) encapsidates the viral genome; the phosphoprotein (P) is the small component of the polymerase; the matrix (M) protein controls host cell gene expression and is involved in the assembly of nucleocapsids; the glycoprotein (G) is embedded in a lipid envelope originating from the infected cell and recognizes specific cellular receptors that allow entry; and the large (L) protein is the main component of the polymerase. VSV is an arbovirus whose life cycle alternates between an arthropod vector and a vertebrate host (14). The virus establishes persistent infections in sand flies and other hematophagous insects and acute infections in mammals, often farm animals such as horses, swine, and cattle. Viral transmission between insects can occur horizontally during cofeeding (15), and infected females can transmit VSV vertically to their offspring (29).

We have previously found that VSV loses fitness in BHK-21 cells when it replicates persistently in sand fly cells, and the fitness loss is due to antagonistic pleiotropy (B. E. Ebendick-Corpus, S. Zárate, J. B. Presloid, and I. S. Novella, submitted for publication). Here we used these low-fitness viral populations to determine the mechanism of recovery once the strains are allowed to replicate in the original BHK-21 mammalian host cells. We determined that recovery was mediated by an increase in the frequencies of highly divergent, minority genomes that coexisted with persistence-adapted strains during persistence. Similar minority subpopulations have been identified in foot-and-mouth disease virus (FMDV) (25) and human immunodeficiency virus type 1 (2), and their survival under unfavorable conditions was termed "memory." Our results demonstrate that these minority VSV genomes constitute a reservoir of variants that emerge rapidly upon appropriate changes in the environment.

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MATERIALS AND METHODS

Cells and viruses. The wild-type (wt) strain that we used is the VSV Indiana serotype of the Mudd-Summers strain, and MARM U is a genetically marked,

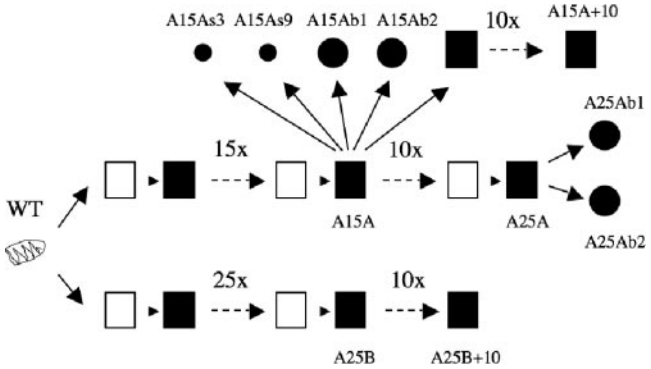

FIG. 1. Evolutionary origins of VSV strains with a history of alternation between persistent insect infection and acute mammalian infection. The wt VSV Indiana serotype (Mudd-Summers strain) is the progenitor of all the evolved populations. White rectangles represent persistent infection of LL-5 sand fly cells; black rectangles represent acute infection of mammalian BHK-21 cells; large circles represent individual big plaques isolated at 20 to 24 h p.i.; little circles represent individual small plaques isolated at 50 h p.i.

antibody-resistant, wt surrogate with a unique substitution in amino acid 259 of the G glycoprotein. Monoclonal antibody I1 hybridoma cells were the generous gift of Douglas Lyles (Wake Forest University) (13). Baby hamster kidney (BHK-21) host cell stocks came from John Holland's laboratory (UCSD), and LL-5 sand fly (*Lutzomyia longipalpis*) cells were kindly donated by Bob Tesh (University of Texas Medical Branch, Galveston) (30). We used minimal essential medium (MEM) supplemented with 0.05% proteose peptone number 3 and 7% heat-inactivated bovine calf serum to grow BHK-21 cell monolayers at concentrations of 0.8 to 1 $\times$ 10^5 cells/cm^2 and Mitsuhashi and Maramorosch (MM) medium (16) supplemented with 20% fetal bovine serum to grow LL-5 cells at 28°C. Virus strains A15A, A25A, and A25B are the result of experimental regimes in which we alternated persistent replication of the wt strain in LL-5 sand fly cells with acute replication in BHK-21 cells, as indicated in Fig. 1 (for details, see reference 34).

Experimental passages. We generated clonal populations of A15A and A25A by diluting the virus stock to a concentration at which we could identify individual, well-isolated plaques. We picked large plaques (>1 mm in diameter) at 20 h postinfection (p.i.), when small plaques were not visible, and small plaques (<1 mm in diameter) at 50 h p.i. (Fig. 1), and we amplified the virus from each plaque by infecting a BHK-21 monolayer at a low multiplicity of infection (MOI), i.e., ≤0.1 PFU/cell. The recovery regimes for alternating A15A and A25B consisted of 10 large-population passages (2 $\times$ 10^5 PFU per passage) at low MOIs in BHK-21 cells (Fig. 1). PerA is the progeny of MARM U after 14 weeks of persistence in LL-5 cells. We infected an LL-5 monolayer with 2 $\times$ 10^5 PFU (MOI, <0.1), and every two weeks, we eliminated detached cells and added fresh medium. PerA + 10 is the progeny of PerA after 10 large-population passages in BHK-21 cells.

Fitness assays. Fitness was determined by direct competition between the test virus and a genetically marked ancestor (MARM U for wt progeny and the wt strain for MARM U progeny). All fitness determinations were done in triplicate single-passage competitions, and fitness is defined as the test/reference ratio after competition divided by the test/reference ratio before competition. These methods have been described in detail elsewhere (7, 11).

Sequencing methods. We have previously reported the sequences of strains A15A, A25A, and A25B (34; Ebendick-Corpus et al., submitted). We obtained the sequences of the remaining evolved strains (A15Ab1, A15Ab2, A15As3, A15As9, A15A + 10, A25Ab1, A25Ab2, A25B + 10, PerA, and PerA + 10) by following methods previously described (19). Briefly, we extracted viral RNA from virus progeny, reverse transcribed it, and used PCR amplification to generate cDNA as a set of 10 overlapping fragments that cover the full length of the viral genome. To resolve the sequences of the 3′ and 5′ termini, we used rapid amplification of cDNA ends. For amplification and sequencing, we used the primers designed by Rodríguez and coworkers (23), with some modifications. The sequences of these primers are available upon request. To determine the order of mutation accumulation, we amplified relevant fragments of intermediate passages. We determined the sequences with an automatic ABI Prism 3730xl sequencer. We assembled the VSV genomes using AssemblyLIGN (Oxford Molecular Group PLC).

Clonal analysis. For clonal analysis, we followed the same procedures as we did for the sequencing of genomes to the step where we obtained purified PCR products. We used primers N1314Fm and M97R to amplify a 1.0-kb band encompassing nucleotides (nt) 1314 to 2349 from alternating populations A15A, A20A, and A25A. The amplified segment has two distinguishing mutations (nt 1377 and 1817). We cloned PCR-amplified molecules into pCR 2.1 (Invitrogen) and picked individual bacterial colonies carrying the plasmid. We tested for the presence of the correct insert, determined the orientation by restriction analysis with XbaI, and sequenced between 88 and 110 plasmids from each population. Sequencing determinations and alignments were done as described above.

RESULTS

Recovery of A15A in BHK-21 cells. A15A is a VSV strain with a history of 15 cycles of persistent replication in sand fly LL-5 cells alternating with acute, cytolytic replication in BHK-21 cells (Fig. 1). As we have previously reported, this regime resulted in a dramatic fitness increase in LL-5 cells and fitness decreases in BHK-21 cells (34). We studied the recovery of population A15A in BHK-21 cells by carrying out 10 passages with large population sizes at low MOIs (see Materials and Methods). When we compared the sequence of the recovered population, A15A + 10 (Fig. 1), with that of its progenitor, A15A, we observed the loss of all of the six point mutations as well as the 43-nt duplication in the 3′ terminus that A15A had accumulated during its evolution from the wt ancestor (Table 1). In addition, we identified six new mutations in the A15A + 10 genome (Table 1). This means that A15A differed from A15A + 10 in a total of 12 point mutations and an insertion. Previous work showed that VSV mutants with similar fitness values generated by repeated bottleneck passages accumulated a single mutation in most cases, and six mutations at most, after 10 recovery passages (19). Moreover, a significant fraction of the bottlenecked mutants did not fix any mutations in that recovery period, despite fitness increases of twofold or more (19); this observation was the result of genomes with beneficial mutations increasing in frequency to a level that was enough to increase the overall fitness of the populations but not enough to produce a peak in the chromatograms of sequencing reactions. These results prompted us to consider the possibility that the recovered A15A + 10 population was not the result of mutations accumulated in the alternating A15A genome. Instead, we hypothesized that the A15A + 10 sequence was present in the A15A population at low frequency.

The A15A population is composed of two clearly different subpopulations. We looked for A15A + 10 sequences in the alternating A15A population by isolating and sequencing individual plaques of large size at 20 to 24 h p.i. We had previously reported variability in the plaque size of A15A when plated on BHK-21 cells, with a majority of small plaques accompanied by occasional larger plaques (34). We assumed that there was some correlation between plaque size and fitness, and that bigger plaques would often represent mammalian cell-adapted strains, while small plaques would usually correspond to persistence-adapted virus with low fitness in BHK-21 cells. Based on these assumptions, we hypothesized that big plaques isolated from A15A at 20 to 24 h p.i. would be the ancestors of A15A + 10. We isolated and sequenced the full-length genomes of two individual large plaques (A15Ab1 and A15Ab2)

TABLE 1. Nucleotide differences between the progenitor wt VSV strain and progeny populations[a]

Nt.	Mut.	Region	AA	A15A Con	A15As3	A15As9	A15Ab1	A15Ab2	A15A+10	A25A Con	A25Ab1	A25Ab2	A25B Con	A25B+10
20	43 Nt.	Leader	na											
163	A→G	N ORF	K34→E											
1377	A→G	IG	na											
1817	C→U	P ORF	T142→M											
2257	C→U	M ORF	S3→Y											
2660	C→U	M ORF	G137											
3104	U→C	G ORF	F9											
3200	G→A	G ORF	P41											
3421	C→A	G ORF	T115→K											
3436	G→C	G ORF	W120→S											
3978	C→A	G ORF	Q301→K											
4122	G→U	G ORF	V349→F											
4139	A→U	G ORF	P354											
5187	A→U	L ORF	K152→I											
6145	A→C	L ORF	N471→P											
8668	C→U	L ORF	T1312→I											
9106	U→C	L ORF	I1458											
9110	G→U	L ORF	D1460→Y											
9950	A→G	L ORF	R1640→G											
10038	C→U	L ORF	S1669→F											

[a] The progeny populations were derived from alternation between persistent infection of LL-5 sand fly cells and acute infection of BHK-21 cells. The history of each strain can be found in Figure 1. The presence of a mutation is indicated by a filled box. Black represents nonsynonymous mutations, dark grey represents synonymous mutations, and light grey represents mutations in noncoding regions. Mut, mutation; AA, amino acid replacement.

(Fig. 1) and observed that all of the mutations characteristic of alternating A15A were absent, while four of the six individual mutations present in recovered A15A + 10 were present (Table 1). In contrast, the sequences of two small plaques isolated at 50 h p.i. (A15As3 and A15As9) (Fig. 1) were very similar to that of alternating A15A, sharing five or six of the seven mutations (including the 3′ duplication). Each small plaque had three additional unique substitutions (Table 1). These results supported our model of the coexistence of two populations during persistence: one majority subpopulation maladapted in BHK-21 cells, represented by the consensus A15A sequence, and a minority subpopulation more fit in BHK-21 cells, represented by the sequence of the large plaques.

The two subpopulations in A15A coexist for an extended period of time. Next we asked whether this mammalian cell-adapted subpopulation would survive after additional persistent passages. We examined this question with two new experiments. In the first experiment, we repeated the analysis of big plaques with alternating population A25A, which is the progeny of A15A after 10 additional cycles of alternating replication (Fig. 1). The consensus sequence of A25A differs from that of A15A in two additional nucleotide substitutions (Table 1). The sequences of two large plaques from A25A (A25Ab1 and A25Ab2) (Fig. 1) included three of the four point mutations found in large A15A plaques and recovered A15A + 10 (Table 1), and this result provided a genetic link between the minority genomes and the recovered strains, in support of our model of extensive coexistence.

In the second experiment, we used a new VSV strain, A25B, generated under the same experimental regime as A25A (Fig. 1). Both strains showed phenotypic and genotypic parallel evolution; the fitness of alternating A25B, like the fitness of A25A, was high in LL-5 cells but low in BHK-21 cells (34). The consensus sequences of the genomes of A25A and A25B share seven point mutations and the 3′ duplication compared to the sequence of their wt ancestor (Ebendick-Corpus et al., submitted). We carried out 10 recovery passages of alternating A25B in BHK-21 cells and determined the sequence of the progeny, A25B + 10 (Fig. 1). The results showed that recovered A25B + 10 lacked all but two of the mutations present in alternating A25B and had five new mutations that were present in the large A15A plaques A15Ab1 and A15Ab2 and the recovered A15A + 10 (Table 1). We can rule out cross-contamination among clones and recovered populations, because there was never overlapping of the times at which we did the experimental passages or the sequence determinations for each population. This result emphasizes the deterministic nature of viral evolution under selective conditions and provides further support to our model of two coexisting populations.

The coexistence of a mammalian cell-adapted subpopulation and an insect cell-adapted subpopulation is not stable in the long term. We considered two models of coexistence. In the first model, the mammalian cell-adapted genomes slowly but steadily decrease in frequency. In the second model, frequency-dependent selection would result in stable coexistence, such that both subpopulations would maintain their relative frequencies for the length of the experiment. We determined the relative frequency of each subpopulation in replica A of the alternating regime at passages 15, 20, and 25 (e.g., A15A, A20A, and A25A) to test which one of the two models was correct. Both the minority and the dominant genotypes are progeny of the wt strain, and therefore, we could not use monoclonal antibody I1 resistance as a means to determine the relative frequencies. Instead, we used clonal analysis of PCR-amplified viral sequences (19). The frequency of the minority (mammalian cell-adapted) subpopulation remained constant

TABLE 2. Nucleotide differences of progeny populations from the progenitor MARM U VSV strain[a]

Nucleotide	62	1913	2108	2151	3224	3281	3846	3853	4210	7748	7774
Mutation	86 nts.	U→C	G→A	C→U	U→C	U→C	G→A	C→A	C→U	A→G	A→A-G
Region	3' Leader	P ORF	M ORF	M ORF	G ORF	G ORF	G ORF	G ORF	G ORF	L ORF	L ORF
Amino acid	na	I→L	G→E	Y	H	I	D→N	A→D	P→L	I→V	I→V
PerA											
PerA+10											

[a] The table shows nucleotide differences in the progeny population after 14 weeks of persistent infection of LL-5 sand fly cells (PerA) and the population after 10 recovery passages in BHK-21 cells (PerA+10). The presence of a mutation is indicated by a filled box. Black represents nonsynonymous mutations, dark grey represents synonymous mutations, and light grey represents mutations in noncoding regions.

at about 10% between passage 15 (8/88) and 20 (9/96) (Pearson's test; $P > 0.8$) but decreased to less than 2% at passage 25 (2/110); this decrease is statistically highly significant (Pearson's test; $P < 10^{-15}$). These results were reasonably consistent with the relative frequencies of the large and small plaques found in alternating A15A and A25A (34). Therefore, the kinetics of mutant frequency supports a model of coexistence that is stable only during a limited period of time.

Periodic replication in mammalian cells is not required for the survival of minority genomes. How can we explain the survival of the mammalian cell-adapted subpopulation during evolution? The periodic steps of mammalian replication must have provided an opportunity for extended survival, but it remained unclear whether these steps were necessary. We then investigated the mechanisms of recovery of a population that had a history of persistence without periodic mammalian replication, PerA. Adaptation of PerA to persistent replication had a cost in BHK-21 cells, such that fitness decreased from 1.0 to 0.23 ± 0.05. PerA differed from its MARM U ancestor in four mutations, including an insertion of 86 nt in position 64 (Table 2). After 10 recovery passages, fitness increased and achieved neutrality (1.09 ± 0.09), and all the mutations accumulated during persistence in PerA were lost in recovered PerA + 10; instead, we identified seven new mutations (Table 2). Thus, we observed the same phenomenon in which a minority subpopulation was maintained during persistence and replaced the persistent population when the virus was returned to BHK-21 cells. We concluded that persistent regimes can support mammalian cell-adapted genotypes at low frequencies and that periodic replication in mammalian cells is not required.

DISCUSSION

Our results demonstrate how RNA virus populations can be more complex than we ever expected, even considering high mutation rates. The VSV minority genomes are reminiscent of memory genomes in FMDV (6). Domingo and coworkers observed that the adaptation of antibody escape, low-fitness mutants to BHK-21 cells was characterized by the preservation of parental sequences at relatively high frequencies (25).

There are three main features shared by minority FMDV and VSV populations: (i) the preservation of memory genomes was reproducible among replicas (27), (ii) the frequency of minority genomes decreased with time, and (iii) the minority genomes accumulated additional changes that contributed to fitness increase and survival in the midst of the newly adapted majority genotypes (1). However, there are also differences.

The two viral species were subjected to very different regimes of adaptation. For FMDV, the regime consisted of relatively low-MOI passages in BHK-21 cells and transmission was horizontal. In contrast, the regimes we used involved persistent replication that presumably promoted consistently high MOIs, and the transmission was strictly vertical, at least in PerA. Also, the frequency of minority genomes in VSV was approximately one order of magnitude higher than that in memory FMDV genomes (25, 26).

The kinetics of VSV minority genomes also differed from the kinetics of FMDV minority genomes in that the latter showed a consistent decrease in frequency through time. VSV minority genomes remained at a constant frequency between passages 15 and 20 in the alternating population replica A, which corresponded to over 2.5 months of replication. The period of coexistence that we observed in these experiments was reasonably consistent with the results from previous work (4, 17). This period of stable coexistence suggests mechanisms promoting frequency-dependent selection (12). It is not surprising that in both FMDV and VSV, the majority genomes eventually prevail, because the competitive exclusion principle precludes indefinite coexistence (32). Another mechanism that would promote survival is the accumulation of beneficial mutations in minority genomes. FMDV C_{22} owed its low fitness to the presence of additional residues in an internal oligo(A) tract (8, 9); the survival of FMDV C_{22} memory genomes was the result of fitness gains likely due to shortening in the oligo(A) tract, but the presence of additional mutations was not investigated (1). In contrast, VSV minority genomes were the result of the wt strain accumulating mutations in unrelated sites.

Our results illustrate a new process that would promote the survival of potentially pathogenic strains and favor their emergence in human populations. Evolutionary theory predicts that specialization has a cost such that adaptation to one host would be generally detrimental to adaptation in other hosts. There is experimental evidence against this prediction (21, 28, 31), but there is also evidence supporting it (5, 10, 31, 33). Thus, we cannot always assume that there will be trade-offs, although they are clearly possible. However, even if trade-offs due to adaptation to a reservoir—such as an animal or a vector—prevent the dominance of variants that are adapted to replication in humans, the survival of minority variants may facilitate species jump upon contact. The same process may take place during antiviral therapy when drug resistance mutations carry a fitness cost. Interruption of antiviral treatment in human immunodeficiency virus type 1 patients may result in the replacement of resistant strains by wt, sensitive strains. However, the dominance of a wt consensus sequence does not

represent the complete loss of resistant mutants, and memory genomes survive at a frequency that is high enough to promote a rapid rebound of resistance when treatment is resumed (2). Note that in this case, there are two types of memory, the cellular memory and the replicative memory. While the latter may be related to FMDV memory and the results reported here for VSV, the former is fundamentally different.

The survival and reemergence of minority genomes can also be a confounding element in studies that use phylogeny to trace the spread of viral strains or to understand the ecology behind viral evolution. For instance, any phylogenetic analysis will place recovered strains closer to the wt strain than their low-fitness, persistent progenitors. This arrangement is formally correct, because the minority genomes did not evolve from the majority genomes; instead, both the minority and the majority genomes evolved simultaneously from the wt strain. However, without the knowledge of the evolutionary history of each strain, we would have concluded that the mammalian cell-adapted, recovered strains were transmitted directly from another infection of mammalian cells, not from the persistently infected insect cells. In a situation where we try to understand the epidemiology of an infection using natural isolates, we would conclude that a strain isolated from an individual (or an animal) was transmitted from another individual (or animal), not from a vector or natural reservoir. Furthermore, the existence of minority genomes is relevant to surveillance efforts, and we propose that clonal analysis of natural isolates will be necessary to identify potential sources of viral pathogens.

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