Many-Trillionfold Amplification of Single RNA Virus Particles Fails To Overcome the Muller's Ratchet Effect

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We showed earlier that transfers of large populations of RNA viruses lead to fitness gains and that repeated genetic bottleneck transfers result in fitness losses due to Muller's ratchet. In the present study, we examined the effects of genetic bottleneck passages intervening between population passages, a process akin to some natural viral transmissions, using vesicular stomatitis virus as a model. Our findings show that the pronounced fitness increases that occur during two successive population passages cannot overcome the fitness decreases caused by a single intervening genetic bottleneck passage. The implications for natural transmissions of RNA viruses are discussed.

Most RNA viruses exhibit a high mutation frequency which is estimated to average about 10^{-4} to 10^{-5} base substitutions per single base site (3, 14, 21, 29, 30, 32, 35, 56, 57, 60). Presumably, this results from viral RNA polymerase infidelity during genome replication and the lack of an error correction mechanism (55). Therefore, for RNA viruses with genomes of approximately 10 kb, many of the progeny may contain one or more mutations. As a result, clonal pools of RNA viruses may consist of a complex mixture of related genomes differing from each other at one or more base sites. These heterogeneous populations are referred to as a quasispecies (6, 15-17, 20). Although a considerable fraction of all mutations may be either lethal or deleterious, a high mutation frequency can be advantageous for RNA viruses whenever there is an extremely large population size. The great genetic diversity and variation present in a large population allow for selection of the fittest variants, and the population as a whole should generally increase in average fitness (8-10, 14, 15, 20, 24, 27, 28, 32, 34, 39, 53, 59, 62). This selection process may allow RNA viruses to escape immune system responses, resist virus inhibitors, and adapt rapidly to new host cell types and environments (14, 28, 31, 39, 47, 50, 61). Also, it may allow extremely debilitated viruses to gain very high fitness (7).

On the other hand, a high mutation rate can be detrimental to asexual RNA viruses if the population is periodically reduced to one or a few virions, a situation which is called a genetic bottleneck (4, 7, 18, 41). Under these conditions, mutation-free genomes become rare and can be lost. For some RNA viruses, recombination may be a means to recreate a mutation-free genome (5, 23, 33, 45, 46). However, in those RNA viruses in which recombination or reassortment is very rare or absent, a mutation-free genome can not be recreated by recombination (25, 48). Thus, the number of deleterious mutations increases in an irreversible ratchet-like manner because of the successive loss of the least-mutated class. Therefore, unless back, compensatory,

or beneficial mutations occur, the population's fitness may decline, a process known as Muller's ratchet (23, 33, 40, 42, 44). Muller's ratchet has been supported by studies of protozoa and in the tripartite RNA bacteriophage $\phi 6$, and we recently observed a wide range of fitness decreases following 20 consecutive plaque-to-plaque transfers of vesicular stomatitis virus, a single-stranded animal RNA virus with an 11-kb genome (2, 4, 7, 18).

RNA viruses may often undergo several rounds of replication in a natural host before one or very few particles initiate a new infection. Therefore, we have now determined whether the biological adaptations occurring during population passages can reverse the stochastic negative effects of Muller's ratchet (4, 7, 18, 28, 39). Three separate experiments were conducted; in each of five transfer series, a different genetically marked monoclonal antibody-resistant mutant (MARM) clone of vesicular stomatitis virus was subjected to 20 cycles of population-population-bottleneck passages at 37°C. The MARM clones were all derived from the Mudd-Summers strain of vesicular stomatitis virus Indiana and have a single amino acid substitution in the major antigenic epitope of the surface glycoprotein which renders them resistant to monoclonal antibody I1 (36-38, 43, 58). Each three-passage cycle was carried out as follows. In population passage 1, virus was picked from a single, well-isolated, randomly chosen plaque and added to 1 ml of minimal essential medium (MEM). From this, 0.2 ml was adsorbed for 20 min at room temperature to a cell monolayer containing 2×10^6 to 3×10^6 BHK or HeLa cells. Next, 6 ml of MEM was added, and the virus was allowed to replicate overnight at 37°C until cytopathic effect was complete. In population passage 2, virus from population passage 1 was harvested and diluted 10,000-fold, and 0.2 ml of the virus was allowed to adsorb and replicate as described above. In the genetic bottleneck passage, the virus yield from population passage 2 was diluted 10 million-fold, and 0.2 ml of the dilution was adsorbed to a fresh monolayer for 15 min at room temperature and then for 35 min at 37°C. The monolayer was then overlaid with 6 ml of MEM containing 0.4% agarose, and plaques were allowed to develop overnight at

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TABLE 1. Statistical analyses of slope differences between experimental and control virus populations

Relative fitness of starting clone	Clone	Slope	Relative fitness (W)	df	F^a	$F_{0.05}^b$	P^c
Very high (Fig. 1A)	Control	1.1287	1.00 ± 0.00				
	Q	-0.4923	0.24 ± 0.01	1 68	676.361	3.989	4.67×10^{-37}
	R	0.6008	0.75 ± 0.01	1 68	40.024	3.993	2.27×10^{-8}
	S	0.3766	0.65 ± 0.02	1 68	32.691	3.989	2.63×10^{-7}
	T	-0.3840	0.29 ± 0.04	1 68	38.664	3.989	3.52×10^{-8}
	U	-0.4984	0.24 ± 0.04	1 68	57.814	3.989	1.32×10^{-10}
Low (Fig. 1B)	Control	-0.0694	1.00 ± 0.00				
	A	-0.1496	0.91 ± 0.01	1 68	37.104	3.989	5.88×10^{-8}
	B	-0.0549	1.02 ± 0.01	1 68	1.040	3.989	0.3114
	C	-0.0857	0.98 ± 0.01	1 65	1.118	3.993	0.2943
	D	0.4095	1.51 ± 0.04	1 68	262.030	3.989	5.09×10^{-25}
	E	-0.3914	0.54 ± 0.03	1 65	155.642	3.993	6.56×10^{-19}
Neutral (Fig. 1C)	Control	-0.0152	1.00 ± 0.00				
	F	0.1818	1.20 ± 0.02	1 68	72.976	3.989	2.27×10^{-12}
	G	-0.2080	0.81 ± 0.02	1 77	113.778	3.977	7.86×10^{-17}
	H	-0.1882	0.82 ± 0.01	1 77	98.373	3.977	2.07×10^{-15}
	I	0.0797	1.10 ± 0.01	1 77	49.267	3.977	7.65×10^{-10}
	J	-0.8295	0.17 ± 0.05	1 77	357.173	3.977	1.20×10^{-80}

^a F , Snedecor F statistic.^b $F_{0.05}$, F statistic according to expected values for 1, 68 or so degrees of freedom.^c P , probability that the observed F value could be obtained at random or that observed F and expected (tabulated) F will be equal.

37°C. This cycle of three passages was repeated 20 times for all 15 passage series, and the relative fitness of the final population in each series was determined by direct competition with wild-type virus during low-multiplicity passages on the indicated cell line (7, 18, 28, 30). The results are presented as fitness vectors which show the replicative abilities of the MARM virus compared with those of wild-type virus (7, 18, 28). The control vector shows the relative fitness of the starting MARM clone, i.e., the fitness of the MARM clone before the experimental passages.

To statistically analyze the fitness differences between the control and the passaged lines, a covariance analysis was conducted (49, 54). The MARM/wild-type virus ratio was log transformed such that the slope of the linear regressions was an estimate of the selection coefficient (i.e., relative fitness) of the corresponding clones (Table 1) (19). The fitness values

(W) of the final MARM populations are shown in Table 1 and were derived from the expression $(1 + s_i)/(1 + s_c)$, where s_i and s_c are the selection coefficients of the passaged and control lines, respectively. As shown in Table 1, except for two clones (clones B and C in Fig. 1B), the selection coefficients of the passaged clones were statistically different from those of their corresponding controls.

Figure 1A shows the results after 20 cycles of alternating population-population-bottleneck passages (60 total passages) starting with a MARM with a very high relative fitness (7, 18, 28). Following the transfers, all the clones showed fitness drops. Clones R and S showed slight declines, while Q, T, and U experienced severe drops. Results of a similar experiment are shown in Fig. 1B, except that the starting MARM clone had previously been subjected to 20 bottleneck passages, lowering its relative fitness (18). After the 20

FIG. 1. Fitness vector diagrams showing the relative fitness of the final MARM population following 20 cycles of population-population-genetic bottleneck passages (60 total passages). Results obtained when the starting clone had a very high relative fitness (A), a low relative fitness (B), and a neutral relative fitness (in this case, the clone was passaged on a new host cell type, HeLa) (C) are shown. The control vector (CON) shows the fitness of the starting virus population, i.e., before 20 cycles of population-population-genetic bottleneck passages. wt, wild type.

population-population-bottleneck passage cycles, clones B and C did not display a significant fitness change, while clone A showed a very slight fitness reduction and clone E experienced a moderate fitness reduction. In contrast to the other findings (but not surprisingly in light of the quasispecies nature of RNA viruses), clone D underwent a dramatic increase in fitness. This higher-fitness clone was probably isolated from the lower-fitness quasispecies population during one of the later genetic bottleneck passages (discussed below). Finally, in the experiment whose results are shown in Fig. 1C, a MARM with a neutral relative fitness was subjected to 20 cycles of population-population-bottleneck passages on HeLa cells, a new host cell type, to determine whether adaptation to a new host cell type could influence our results (28). Two clones, F and I, showed a slight fitness gain; two others, G and H, showed a moderate decline in fitness; and J displayed a severe fitness decline.

Our findings on the consequences of population passages interspersed with bottleneck passages are remarkable because the fitness losses were similar to those obtained after 20 consecutive bottleneck passages alone (7, 18). However, it should be emphasized that the protocol we chose for the experiments was rather stringent because every pair of passages was regularly followed by a clonal bottleneck. Our results would certainly have been different if our bottleneck transfers had involved pooled plaques (e.g., 5 or 20 plaques pooled for each bottleneck) rather than a single virion clone. We are currently investigating whether repetitive bottlenecks composed of five or more clones exhibit Muller's ratchet effect. Likewise, if more than two population passages had intervened between our bottleneck passages, we might have observed different results. Nevertheless, single-virion bottlenecks are probably very common during respiratory transmission of viruses because most respiratory droplets should contain very few or no infectious particles (1, 11, 12, 26). It should also be noted that our two consecutive population passages represented much greater competitive amplification of each cloned virion than would normally occur in a single infected animal or human. Each plaque contains over 10^6 PFU, and each was diluted and amplified to between 10^{11} and 10^{12} PFU and then again diluted 10^4 -fold and amplified to 10^{11} to 10^{12} PFU. This represents many-trillionfold amplification, during which selective competition occurs (7), and demonstrates that Muller's ratchet effects on RNA viruses (4, 5, 18) can persist despite intervening selection of populations. This might be expected when the process starts with a high-fitness population, since a clone of higher fitness is less likely to be isolated. However, it seemed less likely with a low-fitness clone because we showed earlier that population passages rapidly improved the fitness of this clone, in which the starting virus population is debilitated (7, 28). Furthermore, our plaque assays can detect only clones which produce visible plaques; they cannot detect lethal or extremely debilitated mutants.

The loss of fitness observed in two or five clones when a neutral-relative-fitness MARM was subjected to 20 cycles of population-population-genetic bottleneck passages on a new host cell type (HeLa) was also unexpected. We previously showed that this MARM greatly increased its fitness on HeLa cells after only 10 adaptive population passages on HeLa cells.

This result suggests that during natural replication and transmission of RNA viruses, periodic genetic bottleneck transmissions (which must sometimes occur during natural viral transfers) could reduce virus population fitness and, in the case of lethal viruses, reduce host mortality (1, 11, 12,

26). Such effects could thereby facilitate survival of hosts and their progeny and subsequently the viruses they support. However, when viruses are cloned in natural infections, this may occur in thousands of hosts and these bottlenecked progeny may compete with each other.

During natural viral transmissions, variability in pathogenesis may often be caused by periodic genetic bottlenecks following replication of large populations. Periodic disease outbreaks may sometimes occur because a highly fit virus is selected (through a genetic bottleneck) from a lower-fitness quasispecies population (Fig. 1C, clone D) (13). Finally, the findings described above have implications for the development and use of attenuated live virus vaccines (22, 51, 52). Chao's discovery (4) that RNA viruses exhibit Muller's ratchet could have a number of important implications for animal and human virology.

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