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Evolution

The red queen reigns in the kingdom of RNA viruses

(virus fitness/virus quasispecies population evolution)

DAVID K. CLARKE*†, ELIZABETH A. DUARTE*, SANTIAGO F. ELENA‡, ANDRÉS MOYA‡, ESTEBAN DOMINGO§, AND JOHN HOLLAND*¶

*Department of Biology and Center for Molecular Genetics, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0116; ‡Departament de Genetica and Servei de Bioinformatica, Facultad de Biologia, 46100 Burjassot, Valencia, Spain; and §Centro de Biologia Molecular "Severo Ochoa" (Consejo Superior de Investigaciones Cientificas–Universidad Autónoma de Madrid), Cantoblanco, 28049 Madrid, Spain

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ABSTRACT **Two clonal populations of vesicular stomatitis virus of approximately equal relative fitness were mixed together and allowed to compete during many transfers *in vitro* as large virus populations. Eventually, one or the other population suddenly excluded its competitor population, yet both the winners and losers exhibited absolute gains in fitness. Our results agree with the predictions of two major theories of classical population biology; the Competitive Exclusion Principle and the Red Queen's Hypothesis, where (in Lewis Carroll's words) "it takes all the running you can do to keep in the same place."**

A quantitative relative fitness assay has previously been used to demonstrate loss of fitness in RNA virus populations due to Muller's Ratchet (1, 2) and to show gains of fitness by natural selection during virus passages (2). Here, we use the relative fitness assay to follow fitness changes during long-term competition between two vesicular stomatitis virus (VSV) populations that had similar starting fitness. This long-term competition allowed us to test on viruses two major hypotheses of classical population biology: the Competitive Exclusion Principle (3) and the Red Queen's Hypothesis (4).

Most if not all RNA virus populations exist as complex mixtures of genetic and phenotypic variants, often referred to as quasispecies populations (5–10). The genetic heterogeneity within a virus quasispecies population results from a high RNA polymerase error rate—which results in misincorporation frequencies averaging about 10^{-4} to 10^{-5} per base site (10–14, 33) and the apparent absence of any error correction or proofreading mechanism (15). The genetic diversity within a virus quasispecies population facilitates adaptation to and improved fitness on existing or changing environments through natural selection, so long as population sizes are large (16–23). Fitness is defined here as the overall replication and survival ability of VSV in the defined environment of the cell culture system used; it is expressed as the ratio of the progeny produced by the variant under analysis to the progeny of a competing reference wild-type (wt) strain (which is assigned a fitness of 1.0). If genetically diverse virus populations are randomly reduced to one or a few infectious particles [as occurs during droplet transmission of respiratory viruses or cell–cell spread of human immunodeficiency virus and other viruses with low rates of infectious particle production (24–27)], then for probabilistic reasons the resulting new population(s) have reduced fitness. Muller (28) first proposed this phenomenon for asexual populations, and confirming observations have been made during studies of protozoa, the RNA bacteriophage $\phi 6$, and the animal RNA virus VSV (1, 2, 29, 30); consequently, the term Muller's Ratchet is now used to describe stepwise decreases in fitness after successive genetic bottlenecks.

MATERIALS AND METHODS

Cells and Virus. BHK-21 cells were grown as cell monolayers under Eagle's minimum essential medium (MEM) containing 5% (vol/vol) bovine calf serum that had been heat-inactivated for 30 min at 60°C prior to incorporation into MEM. The BHK-21 cells were originally obtained from the American Type Culture Collection. Difco protease peptone 3 was added to MEM at a final concentration of 0.06% to promote rapid BHK-21 cell growth and formation of uniform cell monolayers. Protease peptone was not added to MEM employed for virus replication or virus plaque assay. The Mudd–Summers strain of VSV was cloned and employed in all studies below as wt virus (fitness defined as 1.0) and as cloned monoclonal antibody (mAb) mutant (virus) (MARM) variants of the same wt clone.

Virus Plaque Assays, Relative Fitness Vector Assays, Mouse mAb, and MARM. Virus plaque assays and relative fitness vector assays were done as described (20). Genetically marked (mAb I1-resistant) MARM clones of fitness nearly equal to wt VSV were selected from the cloned wt VSV during earlier studies as described (16, 29). MARM and wt VSV clonal populations were mixed together (passage 0) and starting ratios were carefully quantitated prior to serial replicative competition passages on BHK-21 cells at 37°C (20). MARM plaque-forming units were quantitated under a 0.4% agarose overlay of MEM containing mAb I1 and total VSV plaque-forming units (wt + MARM) were measured by plaque assay in the absence of mAb I1 (20). Triplicate plaque assays were used to generate data points at five-passage intervals. Plaque assays were carried out in 25-cm^2 flasks containing monolayers of BHK-21 cells; 0.2 ml of appropriately diluted supernatant from competition passages was adsorbed to each monolayer for 15 min at room temperature and then for 30 min at 37°C. Each monolayer was then overlaid with 6 ml of MEM containing 0.4% agarose or 6 ml of MEM containing 0.4% agarose and 30% (vol/vol) mAb I1 in MEM (maximal neutralizing level of mAb). After the agarose gelled, the plaques were allowed to develop overnight at 37°C. Competition passages were carried out by adsorption of 0.2 ml of infected cell supernatant (diluted 1:10,000 to eliminate interference by defective interfering particles) to BHK-21 cell monolayers containing 2–3 × 10^6 cells; after virus adsorption (15 min at room temperature and 30 min at 37°C) 7 ml of MEM was added to each monolayer and cell cultures were incubated overnight at 37°C until viral

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Abbreviations: VSV, vesicular stomatitis virus; mAb, monoclonal antibody; MARM, monoclonal antibody-resistant mutant (virus); wt, wild type.

†Present address: Aviron, Inc., 1450 Rollins Road, Burlingame, CA 94010.
¶To whom reprint requests should be addressed.

cytopathology was complete. The average multiplicity of infection at each competition passage was ≈0.1. Each passage was repeated as above and wt/MARM ratios were measured at intervals (20).

RESULTS

Prolonged Replicative Competition of Neutral Fitness Populations. In a few instances, VSV clonal populations resulting from repeated genetic bottlenecks exhibit relative fitness similar to starting virus (1). In the present study, we have continued the virus competitions in three of these cases to examine the nature of the struggle for dominance by each virus population and to see whether the competitive interactions of RNA viruses had parallels in classical population biology. As previously described, each of our competition series contained a mixture of genetically marked MARM virus (clones D, G, and H) and wt virus of similar relative fitness (16, 29). We continued the competition passages for each mixture as before and measured changes in fitness every five passages by triplicate plaque assays carried out in the presence or absence of mAb I1 as described (20). The results of three competition series are presented as fitness vectors that compare the competitive replication abilities of the MARM virus with wt virus.

Fig. 1*A* shows the fitness changes in the competition when wt virus was cultured with clones D, G, and H. The MARM virus (clones D, G, and H) in each of three series displayed very similar fitness levels to each other and to the competing wt virus over the first 12–15 passages. However, in competition series with MARM D, the MARM virus suddenly and completely dominated the culture between passages 23 and 28 such that no wt virus was observed by plaque assay at passage 28. In the competition series with MARM H, the MARM and wt virus population maintained similar fitness for many passages, until at passage 48 MARM H suddenly dominated wt virus. Competition between wt virus and MARM G likewise shows wt and MARM virus populations

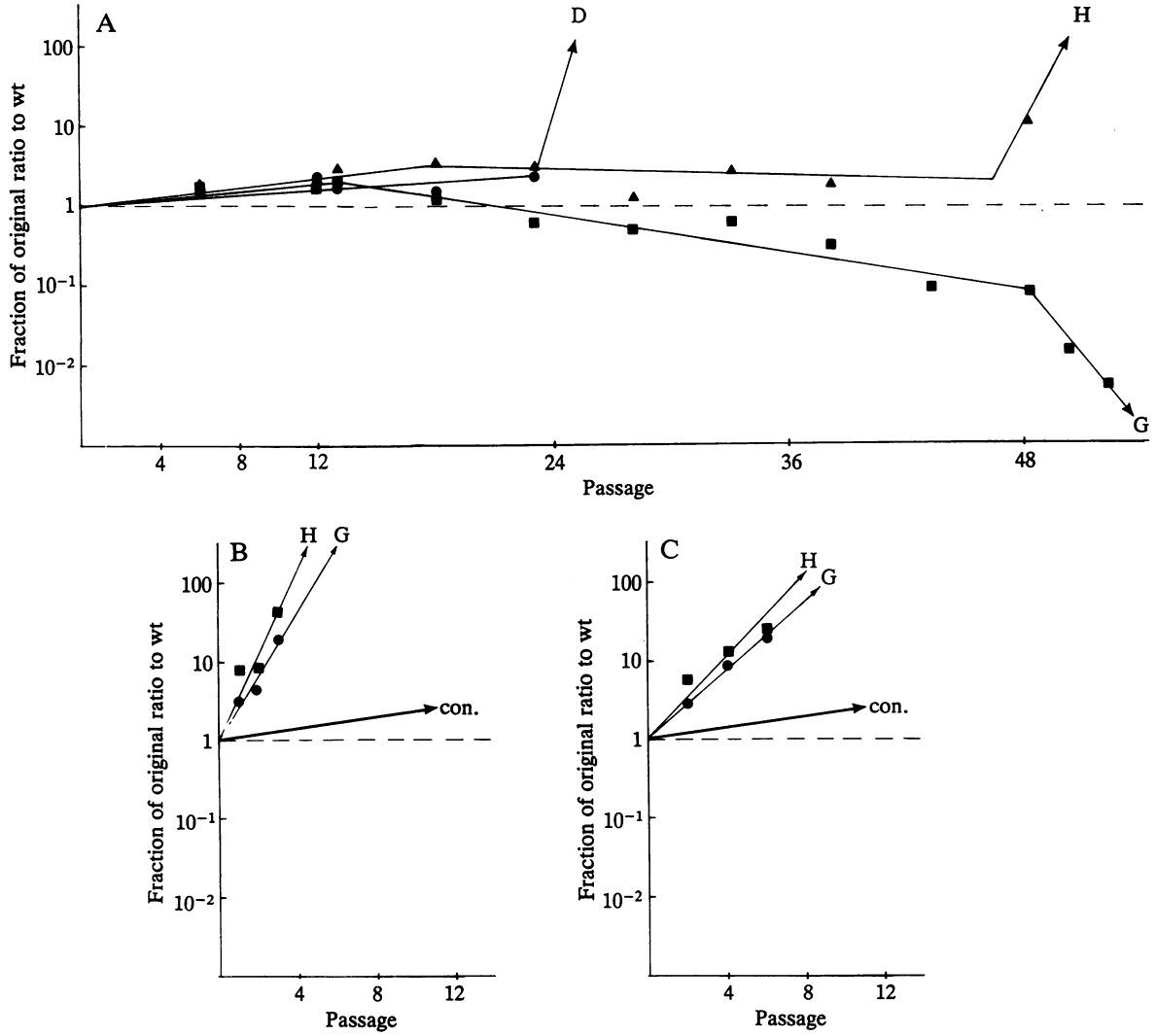

FIG. 1. (*A*) Vector diagram showing the fitness of MARMs D, G, and H relative to wt virus during prolonged competition passages on BHK-21 cells. Vectors were determined as described (20). Competition passages were continued until one or the other competitor dominated. For vector D at passage 33, MARM D had overwhelmed wt virus such that wt was a very small (and therefore indeterminable) fraction of the competition mixture. Similarly, MARM H had overwhelmed wt at passage 53. (*B* and *C*) Vector diagrams showing the relative fitness of winners (MARM H and wt; vectors H and G, respectively) and losers (wt and MARM G; vectors H and G, respectively), respectively, by competition when they were cultured back with virus of starting fitness levels. To do this, winning passage 51 (p51) MARM H was mixed and cultured with wt virus (the same wt VSV clone employed to start the competition series shown in *A*.) Also the winning p51 wt virus of series G was mixed with surrogate wt (MARM C). Finally, the loser p38 wt virus from series H was mixed and cultured with MARM C, and loser p43 MARM G virus was mixed with original starting wt VSV. Control (con.) vector shows the starting fitness of MARM virus relative to wt prior to the long-term competition series of *A*.

Table 1. Relative fitness values before and after stochastic fitness changes during virus competitions

MARM series	Fitness value	
	Before	After
D	0.8181 ± 0.0251	1.0152 ± 0.0303
G	0.7996 ± 0.0289	0.4223 ± 0.0098
H	0.8596 ± 0.0321	1.2602 ± 0.0399

Regression analyses and fitness determination were performed as described (2). Data are the mean ± SEM. Data are from Fig. 1*A*.

maintaining approximately equal fitness for many passages; then at passages 48–50 the wt virus gained a decisive advantage over the MARM G virus population and rapidly dominated the culture. It should be noted for both MARM D and MARM H that by the final competition passages (p33 and p53, respectively), the proportion of wt virus surviving was so small that it could no longer be determined. Statistical analyses and fitness determinations were performed as described (2) except that experimental values were divided into two groups, before and after stochastic fitness jumps. As shown in Table 1, all three series (D, G, and H) exhibited significantly different relative fitness values after stochastic changes. These results show that two virus populations of approximately equal fitness can coexist during prolonged replication in the same stable environment; but stochastic changes in the population balance do eventually occur, and their impact on the competition is sudden and decisive.

In classical population biology, the "Competitive Exclusion Principle" states that in the absence of niche differentiation, one competing species will always eliminate or exclude the other (3). However, competitive exclusion was rejected as a general principle by Ayala (31, 32). His experiments showed that two species of *Drosophila* could coexist while competing for limited resources (31, 32). The very high error frequency of RNA genome replication may make improbable the prolonged coexistence of two genetically distinct viral subpopulations. Variant genomes are constantly generated that may have differing replicative abilities and/or negative effects on competing virus populations. Because extremely high fitness variants arise rather infrequently by combinations of mutations so too the changes in population fitness behave in a stochastic manner.

After the competition series shown in Fig. 1*A*, we decided to culture the winners and losers of competition series G and H back with virus populations of starting fitness levels. To do this we mixed winning passage 51 (p51) MARM H with wt virus (the same wt was used to start the competition series shown in Fig. 1*A*). Also the winning p51 wt virus of series G was mixed with surrogate wt (MARM C), which has almost the same relative fitness as the wt used to start the competition series (20). In addition, the loser p38 wt virus from series H was mixed with MARM C and the loser p43 MARM G virus was mixed with original starting wt. Competition passages and plaque assays of competition mixtures were carried out as before. The results of these competitions are shown in Fig. 1 *B* and *C*, for the winners and losers, respectively. As expected, the winners from series H and G (MARM H virus and wt virus, respectively) show much higher fitness than their starting fitness levels prior to any competition passages. Surprisingly, the losers of competition series H and G (wt and MARM G virus, respectively) also had gained much higher fitness. Table 2 shows that both winners and losers had significantly increased fitness.

Table 2. Relative fitness values for winners and losers

MARM series	Fitness value	
	Winners	Losers
H	1.8191 ± 0.0141	1.2830 ± 0.0331
G	1.6316 ± 0.0306	1.2769 ± 0.0100

Regression analyses and fitness determination were performed as described (2). Data are the mean ± SEM. Data are from Fig. 1 *B* and *C*.

DISCUSSION

Apparently during the long-term replicative competition process there was continuous selection for the most fit genotypes within both competing virus populations; perhaps with one or other virus population playing fitness catch-up at different points in the competition series thus allowing for continuous (and nearly parallel) improvement in fitness of both virus populations for considerable periods. Van Valen (4) proposed a Red Queen's Hypothesis in which each species is competing in a zero-sum game against others; each game is a dynamic equilibrium between competing species where "no species can ever win and new adversaries grinningly replace the losers." In our study, with only two competitor virus populations in each case, only vastly superior mutants derived rather infrequently and stochastically from the quasispecies populations were able to disrupt the status quo causing displacement of one of the virus populations. Presumably, in an animal or human infected by RNA viruses similar processes of near-equal competition followed by displacement also occur, but changes in the adaptive environment (various host cell types, immune and inflammatory response, etc.) might tend to lessen this. Finally, the fact that one population eventually displaces (or is displaced by) its competitor should not obscure the fact that for very long series of virus transfers, during which immense numbers of viruses were produced, two competing virus populations maintained nearly equal fitness by constant running to stay in the same place relative to their competitor. Likewise, due to high mutation rates (6, 8, 15–19), countless mutations occurred during these competition transfers, but only a miniscule fraction could have survived repeated passages and become dominant within the "winner" quasispecies populations. Because of their high mutation rates, rapid replication, large population sizes, and controlled (variable or constant) host cell environments, RNA viruses may be useful subjects for examining other evolutionary hypotheses.

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