JOURNAL OF VIROLOGY, Dec. 1990, p. 6278–6281
0022-538X/90/126278-04$02.00/0

Vol. 64, No. 12

RNA Virus Quasispecies Populations Can Suppress Vastly Superior Mutant Progeny

JUAN CARLOS DE LA TORRE† AND JOHN J. HOLLAND*

Institute of Molecular Genetics and Department of Biology, C-016, University of California at San Diego, La Jolla, California 92093

Received 5 June 1990/Accepted 20 August 1990

A variant clone of vesicular stomatitis virus recovered from a high-passage, evolving virus population replicated rapidly and produced remarkably high yields of virus, but these variants never dominated during further passages. We show that this clone is highly competitive, but it can overwhelm its progenitor population only when seeded above threshold level during dilute passages.

RNA viruses in general exhibit high mutation frequencies, but some lower frequencies have also been reported (2, 4–12, 16, 18, 28–30, 32, 34, 38, 41; J. Coffin, *in* E. Kurstak, R. G. Marusyk, F. A. Murphy, and M. H. V. Van Regenmortel, ed., *Applied Virology Research*, in press). These high mutation frequencies produce heterogeneous quasispecies populations even in virus clones (4, 6, 7, 10, 16, 18, 20, 32–34, 36, 39; Coffin, in press). This facilitates but does not necessitate rapid virus evolution in vitro, as well as in vivo during transmission in nature (2, 3, 5–8, 11, 13–17, 19–23, 25–27, 31, 33–39, 41; Coffin, in press). Clones of vesicular stomatitis virus can exhibit extreme rates of evolution in vitro and in vivo (19, 31, 35, 37, 38). However, they can also exhibit

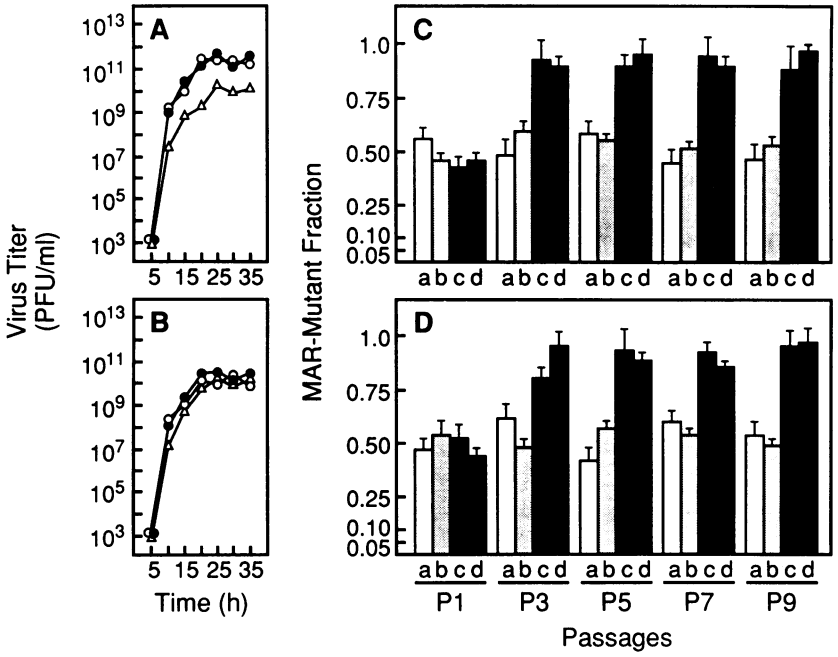

FIG. 1. Comparison of replication kinetics and relative competitive abilities of clonal virus populations 1 and 4 and their corresponding clone 1 MAR and clone 4 MAR populations in BHK_{21} cells in culture at 33°C. (A) One-step growth curves of clone 1 and clone 1 MAR. Symbols: ●, clone 1; ○, clone 1 MAR; △, *ts*G31. (B) One-step growth curves of clone 4 and clone 4 MAR. Symbols: ●, clone 4; ○, clone 4 MAR; △, *ts*G31. (C and D) Competition experiments between clone 1 and clone 1 MAR (panel C), and clone 4 and clone 4 MAR (panel D). Data for competition between clone 1 MAR and *ts*G31 and between clone 4 MAR and *ts*G31 are included for comparison. The different competitions are indicated by letters as follows: clone 1 and clone 1 MAR at low (a) and high (b) multiplicity, and clone 1 MAR and *ts*G31 at low (c) and high (d) multiplicity (panel C); clone 4 and clone 4 MAR at low (a) and high (b) multiplicity, and clone 4 MAR and *ts*G31 at low (c) and high (d) multiplicity (panel D). Selection of clone 1 MAR and clone 4 MAR populations with monoclonal antibody I1 was done as previously described for other MAR variants (40). During the competition experiments, the fraction at each passage of MAR mutant resistant to I1 was determined by using agarose overlays containing I1 to avoid underestimates due to phenotypic masking and/or mixing (18). Low-multiplicity passages for competition studies were done after 10^4-fold dilutions of virus from the previous passage; high-multiplicity passages used undiluted inoculum from the previous passage of virus in BHK_{21} cells.

* Corresponding author.
† Present address: Department of Neuropharmacology, Research Institute of Scripps Clinic, La Jolla, CA 92037.

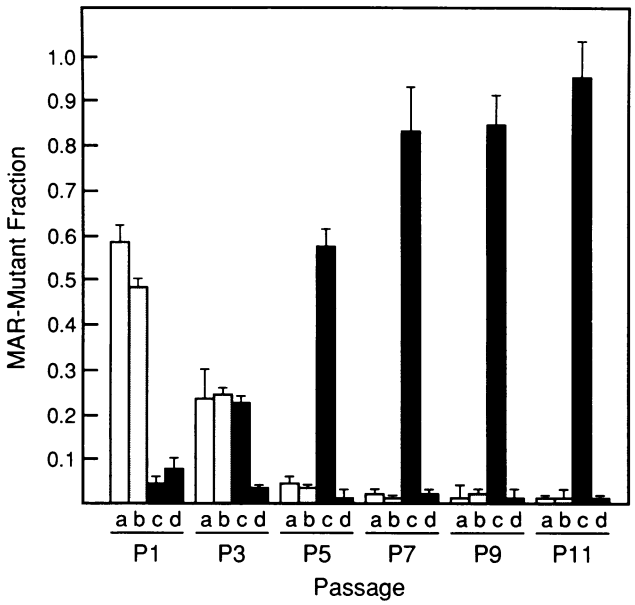

FIG. 2. Competition between clone 1 and clone 4 in BHK_{21} cells at 33°C. Competition passages were done at high and low multiplicity as for Fig. 1. The different competition experiments are indicated by letters as follows: clone 1 and clone 4 MAR, equal passage 1 inputs, at low (a) and high (b) multiplicity; clone 1 MAR and clone 4 (ratio 0.1:0.9) at low multiplicity (c); clone 1 and clone 4 MAR (ratio 0.9:0.1) at low multiplicity (d). Methods are as described in the legend to Fig. 1.

prolonged genetic stability (35, 37) when passaged under conditions (such as dilute passages) which promote relative population equilibrium (i.e., prolonged dominance of high-fitness "master sequences"). Very little is known of the virus population dynamics by which complex quasispecies (11) populations evolve or maintain equilibrium.

During serial undiluted (high-multiplicity) passages at 33°C, vesicular stomatitis virus undergoes rapid and continuous evolution (35). We recently sampled the quasispecies population of genomes present at passage 254 by isolating a number of clones (37). Two of these, clone 1 and clone 4, exhibited superior fitness relative to their original progenitor virus, *ts*G31, and both were present as a rather low proportion of the population at passage 254 (37). However, clone 1 exhibited remarkably high yields and very rapid one-step growth curves compared with *ts*G31, wild-type vesicular stomatitis virus, or clone 4 (37). To study the population dynamics of these two clones, we have marked them with selectable genetic traits by picking monoclonal antibody-resistant (MAR) clones of each by using the I1 monoclonal antibody (24, 40). The introduction of this genetic marker did not greatly alter the yields of clone 1 MAR (ca. 10^{11} PFU/ml and 20 particles per PFU) or of clone 4 MAR (ca. 2×10^9 PFU/ml and 80 particles per PFU). Figure 1A and B compare the replication kinetics (in one-step growth experiments) of cone 1, clone 1 MAR, clone 4, clone 4 MAR, and their progenitor, *ts*G31. Clearly, the rate and extent of replication of clone 1 and clone 1 MAR exceed those of clone 4 and clone 4 MAR (which only slightly exceeds *ts*G31 in replication efficiency). The monoclonal antibody resistance marker on clone 1 MAR did not alter its competitive ability (Fig. 1C). When mixed with clone 1 at a ratio of approximately 0.5, clone 1 MAR maintained this starting fraction throughout nine dilute (a) or undilute (b) passages in BHK_{21} cells. In competition studies with *ts*G31, both clone 1 and clone 1 MAR quickly dominated the population by the third passage (c and d). Figure 1D shows similar results for clone 4 and clone 4 MAR.

Having demonstrated that clone 1 MAR and clone 4 MAR

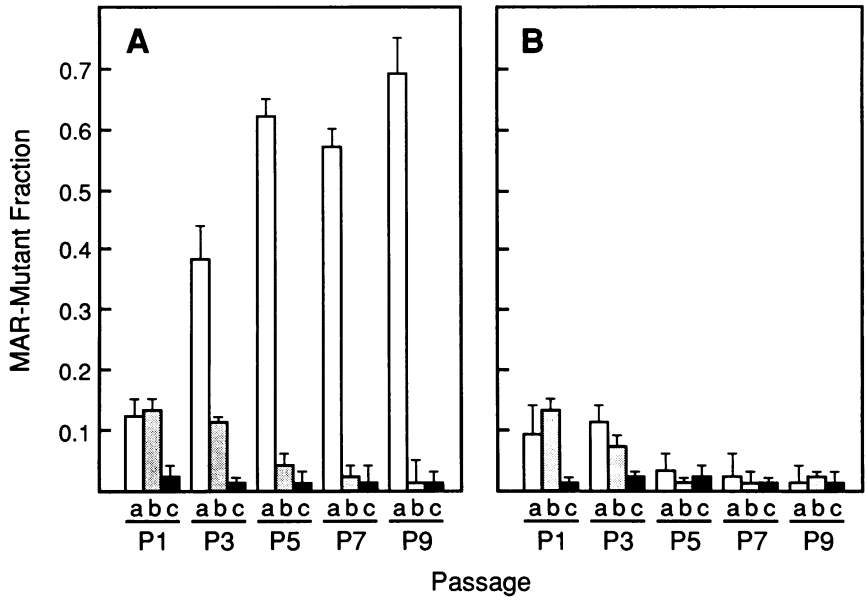

FIG. 3. Competition between clones 1 and 4 and the progenitor total virus population (passage 254) in BHK_{21} cells at 33°C. (A) Competition between clone 1 MAR and passage 254 virus, with a clone 1 MAR input fraction of 0.1 at low (a) and high (b) multiplicity and with a clone 1 MAR input fraction of 10^{-3} at low multiplicity (c). (B) Competition between clone 4 MAR and passage 254 virus, with a clone 4 MAR input fraction of 0.1 at low (a) and high (b) multiplicity and with a clone 4 MAR input of 10^{-3} at low multiplicity (c). Parallel control passages of passage 254 virus alone or clone 1 or 4 alone showed that mutationally derived MAR mutants did not increase above 10^{-4} during nine passages of passage 254 virus, nor did monoclonal antibody-sensitive revertants displace clone 1 MAR or clone 4 MAR during nine passages.

replicate and compete similarly to their unmarked progenitor clones, we next analyzed their ability to compete with each other. Clone 1 rapidly outcompeted clone 4 (Fig. 2). This occurred whether the MAR marker was on clone 4 (a and b) or clone 1 (c and d) and occurred during both dilute and undiluted passage series. This was the expected result because clone 1 produces very large yields and replicates more rapidly than clone 4 (Fig. 1). Finally, we examined the competitive characteristics of clone 1 MAR and clone 4 MAR when they were seeded back into the complex quasispecies population from which they had been isolated after 254 serial undiluted passages in BHK_{21} cells (35, 37). Figure 3 shows the results obtained during serial passages in BHK_{21} cells after clone 1 MAR (A) or clone 4 MAR (B) was seeded into the total passage 254 virus population at starting fractions of 10^{-1} or 10^{-3} of total virus PFU. The fraction of MAR mutant was measured under I1 monoclonal antibody in agarose overlays to circumvent phenotypic mixing (18) after both dilute and undiluted passages of virus. Clone 4 MAR was strongly outcompeted under all conditions (Fig. 3B) by the total passage 254 virus population. In contrast, the competition fate of clone 1 MAR depended upon the level at which it was initially seeded into the mixed virus population prior to the first passage in a dilute-passage series (Fig. 3A). With a starting fraction of 10^{-1} (a) it rose to dominance by passage 5 and thereafter, but with a starting fraction of 10^{-3} (c) it was quickly outcompeted by its complex progenitor passage 254 virus population. Finally, even at a starting fraction of 10^{-1}, clone 1 MAR could not compete with its complex progenitor passage 254 virus (and defective interfering particle) population during undiluted passage series in BHK_{21} cells [Fig. 3A (b)].

The above results demonstrate that even virus variants of remarkable relative fitness may be hidden and suppressed within a complex quasispecies population of lower average relative fitness. The emergence of potentially dangerous new virus pathogens in nature may often be prevented by such population dynamics. Occasional emergence of novel pathogens may depend upon low-probability bottleneck transmissions in which the suppressed new variant is transmitted as a single particle or as one of several particles, thus exceeding a threshold limitation. Finally, such population dynamics may often confound efforts to correlate observed phenotypic effects (such as disease syndrome, tissue pathology, etc.) with single genome variants of defined sequence. The complex mixture of virus (and defective interfering particle) variants present in the passage 254 population (35, 37) cannot be analyzed completely. This requirement of thresholds for dominance (or even detection) dictates an element of uncertainty in virus sampling during outbreaks in nature.

This work was supported by Public Health Service grant AI 14627 from the National Institutes of Health. J.C.T. is a Fulbright Postdoctoral Fellow.

LITERATURE CITED

1. **Air, G. M., A. J. Gibbs, W. G. Laver, and R. G. Webster.** 1990. Evolutionary changes in influenza B are not primarily governed by antibody selection. Proc. Natl. Acad. Sci. USA **87**:3884–3888.
2. **Batschelet, E., E. Domingo, and C. Weissman.** 1976. The proportion of revertant and mutant phage in a growing population as a function of mutation and growth rate. Gene **1**:27–32.
3. **Cattaneo, R., A. Schmid, D. Eschle, K. Baczko, V. ter Meulen, and M. A. Billeter.** 1988. Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. Cell **55**:255–265.
4. **de la Torre, J. C., E. Wimmer, and J. J. Holland.** 1990. Very high frequency of reversion to guanidine resistance in clonal pools of guanidine-dependent type I poliovirus. J. Virol. **64**:664–671.
5. **Domingo, E., M. Dávilla, and J. Ortín.** 1980. Nucleotide sequence heterogeneity of the RNA from a natural population of foot-and-mouth disease virus. Gene **11**:333–346.
6. **Domingo, E., R. A. Flavell, and C. Weissmann.** 1976. *In vitro* site directed mutagenesis: generation and properties of an infectious extracistronic mutant of bacteriophage Qβ. Gene **1**:3–25.
7. **Domingo, E., and J. J. Holland.** 1988. High error rates, population equilibrium and evolution of RNA replication systems, p. 3–36. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics. CRC Press, Inc., Boca Raton, Fla.
8. **Domingo, E., D. Sabo, T. Taniguchi, and C. Weissman.** 1978. Nucleotide sequence heterogeneity of an RNA phage population. Cell **13**:735–744.
9. **Durbin, R. K., and V. Stollar.** 1986. Sequence analysis of the E1 gene of a hyperglycosylated, host restricted mutant of Sindbis virus and estimation of mutation rate from frequency of revertants. Virology **154**:135–143.
10. **Eggers, H. J., and I. Tamm.** 1965. Coxsackie A9 virus: mutation from drug dependence to drug resistance. Science **148**:97–98.
11. **Eigen, M., and C. K. Biebricher.** 1988. Sequence space and quasispecies distribution, p. 211–245. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics. CRC Press, Inc., Boca Raton, Fla.
12. **Fields, B. N., and W. K. Joklik.** 1969. Isolation and preliminary characterization of temperature sensitive mutants of reovirus. Virology **37**:335–342.
13. **Gebauer, F., J. C. de la Torre, I. Gomez, M. G. Mateu, H. Barahoma, B. Teraboschi, I. Bergmann, P. Augé de Mello, and E. Domingo.** 1988. Rapid selection of genetic and antigenic variants of foot-and-mouth disease virus during persistence in cattle. J. Virol. **62**:2041–2049.
14. **Gojobori, T., and S. Yokoyama.** 1985. Rates of evolution of the retroviral oncogene of Moloney murine sarcoma virus and of its cellular homologues. Proc. Natl. Acad. Sci. USA **82**:4198–4201.
15. **Gorman, O. T., W. J. Bean, Y. Kawaoka, and R. G. Webster.** 1990. Evolution of the nucleoprotein gene of influenza A virus. J. Virol. **64**:1487–1497.
16. **Granoff, A.** 1964. Nature of the Newcastle disease virus population, p. 107–108. *In* R. P. Hanson (ed.), Newcastle disease virus, an evolving pathogen. University of Wisconsin Press, Madison, Wis.
17. **Hahn, B. H., G. M. Shaw, M. E. Taylor, R. R. Redfield, P. D. Markham, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, E. S. Parks, and W. P. Parks.** 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. Science **232**:1548–1553.
18. **Holland, J. J., J. C. de la Torre, D. A. Steinhauer, D. Clarke, E. Duarte, and E. Domingo.** 1989. Virus mutation frequencies can be greatly underestimated by monoclonal antibody neutralization of virions. J. Virol. **63**:5030–5036.
19. **Holland, J. J., E. A. Grabau, C. L. Jones, and B. L. Semler.** 1979. Evolution of multiple genome mutations during long term persistent infection by vesicular stomatitis virus. Cell **16**:494–504.
20. **Holland, J. J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol.** 1982. Rapid evolution of RNA genomes. Science **215**:1577–1585.
21. **Huovilainen, A., T. Hori, L. Kinnunen, K. Takkinen, M. Ferguson, and P. Minor.** 1987. Evolution of poliovirus during an outbreak: sequential type 3 poliovirus isolates from several persons show shifts of neutralization determinants. J. Gen. Virol. **68**:1373–1378.
22. **Kew, O., M. B. K. Nottay, M. H. Hatch, J. H. Nakano, and J. F. Obijeski.** 1981. Multiple genetic changes can occur in the oral poliovaccines upon replication in humans. J. Gen. Virol. **56**:337–347.
23. **Kinnunen, L., A. Huovilainen, T. Pöyry, and T. Hovi.** 1990. Rapid molecular evolution of wild type poliovirus during infection in individual hosts. J. Gen. Virol. **71**:317–324.
24. **Lefrancois, L., and D. Lyles.** 1982. The interaction of antibody

with the major surface glycoproteins of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies. Virology **121**:168–174.
25. **Minor, P. D., A. John, M. Ferguson, and J. P. Icenogie.** 1986. Antigenic and molecular evolution of the vaccine strain of type 3 poliovirus during the period of excretion by a primary vaccine. J. Gen. Virol. **67**:693–706.
26. **Nichol, S. T.** 1988. Genetic diversity of enzootic isolates of vesicular stomatitis virus New Jersey. J. Virol. **62**:572–579.
27. **Nichol, S. T., J. E. Rowe, and W. M. Fitch.** 1989. Glycoprotein evolution of vesicular stomatitis virus New Jersey. Virology **168**:281–291.
28. **Pincus, S. E., D. C. Diamond, E. A. Emini, and E. Wimmer.** 1986. Guanidine-selected mutants of poliovirus: mapping of point mutations to polypeptide 2C. J. Virol. **57**:638–646.
29. **Portner, A., R. G. Webster, and W. Bean.** 1980. Similar frequency of antigenic variants in Sendai, vesicular stomatitis virus, and influenza A viruses. Virology **104**:235–238.
30. **Rowlands, D. J., B. E. Clarke, A. R. Carroll, F. Brown, B. H. Nickolson, J. L. Bittle, R. A. Houghten, and R. A. Lerner.** 1983. Chemical basis of antigenic variation in foot-and-mouth disease virus. Nature (London) **306**:694–697.
31. **Saag, M. S., B. H. Hahn, J. Gibbons, Y. Li, E. S. Parks, W. P. Parks, and G. M. Shaw.** 1988. Extensive variation of human immunodeficiency virus type-1 *in vivo*. Nature (London) **334**: 440–444.
32. **Sedivy, J. M., J. P. Capone, U. L. Raj Bhandary, and P. A. Sharp.** 1987. An inducible mammalian amber suppressor: propagation of a poliovirus mutant. Cell **50**:379–389.
33. **Smith, D. B., and S. C. Inglis.** 1987. The mutation rate and variability of eukaryotic viruses: an analytical review. J. Gen. Virol. **68**:2729–2740.
34. **Smith, F. I., and P. Palese.** 1988. Influenza virus: high rates of mutation and evolution, p. 123–135. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics. CRC Press, Inc., Boca Raton, Fla.
35. **Spindler, K. R., F. M. Horodyski, and J. J. Holland.** 1982. High multiplicities of infection favor rapid and random evolution of vesicular stomatitis virus. Virology **119**:96–108.
36. **Steinhauer, D. A., J. C. de la Torre, and J. J. Holland.** 1989. High nucleotide substitution error frequencies in clonal pools of vesicular stomatitis virus. J. Virol. **63**:2063–2071.
37. **Steinhauer, D. A., J. C. de la Torre, E. Meier, and J. J. Holland.** 1989. Extreme heterogeneity in populations of vesicular stomatitis virus. J. Virol. **63**:2072–2080.
38. **Steinhauer, D. A., and J. J. Holland.** 1987. Rapid evolution of RNA viruses. Annu. Rev. Microbiol. **41**:409–433.
39. **Strauss, J. H., and E. G. Strauss.** 1988. Evolution of RNA viruses. Annu. Rev. Microbiol. **42**:657–683.
40. **VandePol, S., L. Lefrancois, and J. J. Holland.** 1986. Sequences of the major antibody binding epitopes of the Indiana serotype of vesicular stomatitis virus. Virology **148**:312–325.
41. **Wong-Staal, F.** 1988. Variation of the HIV genome: implications for the pathogenesis and prevention of AIDS, p. 147–157. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics. CRC Press, Inc., Boca Raton, Fla.