Journal of Virology, Sept. 1989, p. 4107–4109
0022-538X/89/094107-03$02.00/0
Copyright © 1989, American Society for Microbiology
Vol. 63, No. 9

Phenotypic Hiding: the Carryover of Mutations in RNA Viruses as Shown by Detection of *mar* Mutants in Influenza Virus

JUAN VALCÁRCEL AND JUAN ORTÍN*

Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

Received 28 December 1988/Accepted 17 May 1989

When influenza virus monoclonal antibody-resistant (*mar*) mutants are selected by incubation in vitro with excess antibody, 90 to 99% of the mutants are not detectable. This observation may be explained by encapsidation of *mar* mutant RNAs within phenotypically wild-type envelopes. This phenotypic hiding can be revealed by selection of *mar* mutants in vivo after virus uncoating. Using experimental procedures appropriate to detect all viable *mar* mutants in a virus population, we determined precisely the mutation rates to the *mar* genotype by the fluctuation test for two nonoverlapping monoclonal antibodies.

The production of monoclonal antibodies (MAbs) specific for viral particles has allowed the detailed characterization of the corresponding antigens (4, 6–8, 11, 12, 15, 21–28), including the definition of the epitopes relevant for neutralization (6, 11, 15, 22, 26), and has provided a means to study the antigenic heterogeneity and evolution of virus populations (3, 16, 26, 28). The frequency with which MAb-resistant (*mar*) mutants are isolated from cloned virus stocks has been used to infer the genetic variability of the virus (19, 23). However, several caveats, both technical and theoretical, have to be taken into account in doing such extrapolation (23). First, the concentration of MAb used must be sufficient to completely block replication of wild-type virus and should not inhibit replication of resistant variants. Second, the possibility of an MAb-mediated inhibition of phenotypically mixed virus replication has to be considered. Third, different amino acid changes at a given epitope may not be biologically equivalent; some may be lethal, whereas others may be irrelevant for virus replication (10). Fourth, the mutability at different gene positions may be different (25), and it may be different for virus particles carrying defective polymerase genes (20). In our approach to use the mutation rate to the *mar* genotype as a means to compare the genetic variability among influenza viruses, we observed that most of the *mar* mutants were hidden by phenotypic mixing, a result that may have important implications in the maintenance of mutations in RNA viruses. These results and their use in determining the mutation rate to *mar* genotypes are the subject of this report.

To optimize the conditions for determining the presence of *mar* mutants in a virus population, we used two alternative experimental procedures. Procedure A included a neutralization step of the viral sample with the appropriate dilution of MAb. The sample was then allowed to adsorb onto an MDCK cell monolayer, and plaques were assayed as described previously (24), except for the presence of MAb in the agar overlay to ensure that the growing plaques were due to *mar* mutants. Procedure B involved the infection of an MDCK monolayer with an appropriate amount of virus. The cultures were incubated for 2 h at 37°C, and then an agar overlay containing the MAb was added. In each case, the amount of MAb used was in excess and did not interfere with the replication of a set of (previously isolated) *mar* mutants (data not shown). Influenza virus A/Victoria/3/75 (VIC) was plaque purified twice and used to prepare virus stocks by expansion from a single PFU to 2×10^5 to 2×10^9 PFU, depending on the sample. The proportion of *mar* mutants in each one was then determined by procedure A and/or procedure B by using nonoverlapping MAbs M58/p7/c (p7) and M234/2/G10 (G10) (13). A large proportion of the plaques isolated by either procedure was characterized as *mar* by plaque assay in the absence or presence of the MAb (data not shown). The results (Table 1) indicate a systematic difference in the proportions obtained by either procedure.

The essential difference between procedures A and B is that the former screens the phenotype of the virions in the population, whereas the latter detects the genotype of the infectious virus in the sample. Hence, a possible explanation for the differences shown in Table 1 is the presence of a large proportion of *mar* mutants with a phenotypically mixed envelope, which would be neutralized by procedure A but not by procedure B, in spite of the fact that all virus stocks tested were prepared at a low multiplicity of infection. In fact, during the packaging of a *mar* mutant RNA, most of the hemagglutinin molecules incorporated in the virion would be wild type. If this virus particle does not enter a new cycle of infection, or if it does so at high multiplicity, then the mutation would remain hidden by the phenotypically mixed envelope. To test this possibility, a reconstruction experiment was performed in which MDCK cells were infected with a mixture of VIC virus and p7RVI, a p7-specific *mar* mutant, in a 10^4:1 proportion, and progeny virus was recovered at several time points in the progression of cytopathic effect. The proportion of *mar* mutant was determined by either procedure A or B (Table 2). It is clear that procedure A heavily underestimates the number of *mar* mutants in the progeny, as compared with the results obtained with procedure B. Furthermore, the values obtained by the latter fit reasonably well with the proportion of *mar* mutants expected, on the assumption that neither genotype has a selective advantage during expansion.

In view of the above results, we considered it technically feasible to detect all *mar* mutants present in a given influenza virus population; hence we applied the fluctuation test (14) to determine the mutation rate to viable *mar* genotype. Statistical analysis of the Poisson distribution predicts that using a sample size of 10 virus stocks with $P(0) = 0.5$ the value obtained for the mutation rate would be accurate within

* Corresponding author.

TABLE 1. Estimation of the proportion of *mar* mutants in a population of VIC influenza virus

MAb	Proportion of *mar* mutant[a] (avg ± SD)	
	Procedure A	Procedure B
p7	$5.4 \times 10^{-6} \pm 9.1 \times 10^{-6}$[b]	$3.3 \times 10^{-5} \pm 2.5 \times 10^{-5}$[c]
G10	$5.5 \times 10^{-7} \pm 6.5 \times 10^{-7}$[d]	$3.3 \times 10^{-5} \pm 1.6 \times 10^{-5}$[e]

[a] Influenza virus VIC stocks were prepared by amplification of a single PFU. For neutralization by procedure A, a sample of the stock (usually 2×10^7 PFU) was mixed with an equal volume of a 10^{-2} dilution of an ascites fluid preparation of the MAb. After incubation for 30 min at 37°C, the mixture was plated onto a MDCK monolayer, and an agar overlay containing the MAb preparation diluted to 10^{-3} was added. Procedure B involved the infection of an MDCK monolayer with a sample of the virus stock (usually 5×10^4 to 5×10^5 PFU for a 100-mm petri dish). After incubation for 2 h at 37°C, an agar overlay was added that contained a 10^{-3} dilution of the MAb preparation. Virus plaques were either counted unstained and picked, or stained with crystal violet and counted. Picked plaques were characterized as *mar* by procedure B. For both procedures, the concentration of MAb used was in excess and did not reduce unspecifically the titer of *mar* mutants.
[b] Value for five determinations.
[c] Value for 14 determinations.
[d] Value for seven determinations.
[e] Value for 24 determinations.

±50%, with a 90% confidence interval. Therefore we expanded 10 independent PFU of VIC virus to develop a plaque (stock size, $\sim 10^5$ PFU) and determined the presence or absence of *mar* mutants by procedure B. The results obtained in five such tests for the site defined by MAb G10 and three tests for that defined by MAb p7 are presented in Table 3. The mutation rates obtained, $3.15 \times 10^{-5} \pm 0.93 \times 10^{-5}$ for site G10 and $5.83 \times 10^{-6} \pm 1.22 \times 10^{-6}$ for site p7, show a dispersion that conforms to the statistical predictions and are clearly distinguishable. This is not surprising, since the probability of mutation to resistance may differ among two different MAb-binding sites according to the number and affinity of the protein-protein contacts, the mutational possibilities at each amino acid position, the effect of each amino acid change on the neutralization by the MAb, the viability of the mutant, etc.

It should be stressed that the measured mutation rates are not rates per nucleotide position and therefore cannot be compared with other values reported previously for RNA virus systems: total mutation rate for a given position in bacteriophage $Q\beta$ RNA (1) and viable mutation rate for influenza virus and poliovirus genes (18). To estimate the mutation rate per nucleotide position, the number of nucleotides whose change may lead to resistance should be determined. Experiments are in progress with such an aim.

TABLE 2. Estimation of the proportion of *mar* mutants in the progeny of a mixed infection with VIC and p7RVI influenza viruses[a]

Cytopathic effect (%)	Proportion of *mar* mutants		
	Predicted	Procedure A	Procedure B
5	8.8×10^{-5}	2.8×10^{-6}	1.6×10^{-4}
40	8.8×10^{-5}	1.4×10^{-6}	1.5×10^{-4}
100	8.8×10^{-5}	2.0×10^{-6}	2.9×10^{-4}

[a] An MDCK monolayer containing 10^7 cells was infected with a mixture of 1.7×10^5 PFU of VIC virus and 15 PFU of p7RVI virus, and samples were taken at the indicated levels of cytopathic effect. Total virus was determined by plaque assay, and titers of *mar* mutants were determined by procedures A and B as described in footnote *a* of Table 1. The predicted proportion of *mar* mutant was calculated from the input virus, assuming no selective advantage of either wild-type or *mar* virus.

TABLE 3. Determination of mutation rate to *mar* genotype at sites defined by MAbs G10 and p7

MAb	Virus population size (PFU)	P(0)[a]	μ[b]
G10	2.5×10^4	0.4	2.77×10^{-5}
	2.5×10^4	0.6	2.04×10^{-5}
	3.5×10^4	0.2	4.60×10^{-5}
	3.0×10^4	0.4	3.05×10^{-5}
	2.7×10^4	0.4	3.30×10^{-5}
p7	5.0×10^4	0.8	4.5×10^{-6}
	1.0×10^5	0.5	6.9×10^{-6}
	1.5×10^5	0.4	6.1×10^{-6}

[a] Determined by procedure B on 10 stocks of virus generated from a single PFU to the size of a plaque.
[b] Calculated from $\mu = -\ln P(0)/N$, assuming that N (number of replication rounds) is approximately equal to the final number of virions. If an amplification of 100 PFU per infective particle occurs in each replication cycle, the number of replication rounds before the last viral generation is ~1% of total replication rounds.

In addition, it should be determined whether any of the possible changes would be lethal and whether the viable mutations are neutral. In this respect, it is worth mentioning that many of the amino acids located in the regions important for neutralization (26) have changed in the course of evolution of the H3 subtype in nature or through in vitro selection (2), suggesting that the viability of *mar* mutants would not be significantly impaired. Furthermore, using experiments similar to that of Table 2 and serial passage at low multiplicity of infection, we analyzed the progeny of mixed infections with wild-type virus and each of a panel of p7 and G10 *mar* mutants. In most instances, the wt/*mar* mutant ratio reflected the input ratio (data not shown). These results suggest that the *mar* genotype is not systematically associated with a decrease in fitness. It could be argued that the mutation rate is in fact higher than was determined but that most of the *mar* mutants are lost by competition in the amplification process. However, most of the *mar* mutants would be produced in the last replication cycle during the development of a plaque, and therefore they would not have had the opportunity to compete further.

In conclusion, we have shown that most of the *mar* mutants in an influenza virus stock are hidden within phenotypically mixed envelopes. Although in the case of a *mar* mutation this situation leads to the elimination of the virus in an immune environment, most other mutations would be carried over to the next host cell. This phenomenon may be at the basis of the genetic polymorphism of influenza viruses (17) and RNA viruses in general (5, 9). Using experimental conditions that presumably allow detection of all viable *mar* mutants in an influenza virus population, we measured the mutation rate to the *mar* genotype by the fluctuation test. Although they do not represent mutation rates per nucleotide position, the values are accurate and reproducible enough to allow faithful comparison of genetic variability among influenza viruses.

We are indebted to E. Domingo, L. Enjuanes, J. A. Melero, G. Morata, and A. Portela for fruitful discussions and suggestions on the manuscript. MAb preparations were generously provided by J. A. Melero. The technical assistance of C. Martínez, J. Rebelles, and F. Ocaña and the secretarial work of C. Hermoso are gratefully acknowledged.

J.V. is a predoctoral fellow of Fondo de Investigaciones Sanitarias. This work was supported by Comisión Asesora de Investigación Científica y Técnica (grant 438) and institutional grants from Fondo de Investigaciones Sanitarias and Fundación Areces.

ADDENDUM IN PROOF

Similar phenotypic hiding of *mar* mutants has been observed in the vesicular stomatitis virus system by J. Holland and colleagues (J. Holland et al., submitted for publication).

LITERATURE CITED

1. **Batchlelet, E., E. Domingo, and C. Weissmann.** 1976. The proportion of revertant and mutant phage in a growing population, as a function of mutation and growth rate. Gene **1**:27–32.
2. **Both, G. W., and M. J. Sleigh.** 1981. Conservation and variation in the hemagglutinins of Hong Kong subtype influenza viruses during antigenic drift. J. Virol. **39**:663–672.
3. **Both, G. W., M. J. Sleigh, N. J. Cox, and A. P. Kendal.** 1983. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. J. Virol. **48**:52–60.
4. **Caton, A. J., G. G. Brownlee, J. W. Yewdell, and W. Gerhard.** 1982. The antigenic structure of the influenza virus A/PR/8/34 hemagglutinin (H1 subtype). Cell **31**:417–427.
5. **Domingo, E., E. Martínez-Salas, F. Sobrino, J. C. de la Torre, A. Portela, J. Ortín, C. López-Galindez, P. Pérez-Breña, N. Villanueva, R. Nájera, S. Vande Pol, D. Steinhauer, N. DePolo, and J. Holland.** 1985. The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance. Gene **40**:1–8.
6. **Emini, E. A., S. Kao, A. J. Lewis, R. Crainic, and E. Wimmer.** 1983. Functional basis of poliovirus neutralization determined with monospecific neutralization antibodies. J. Virol. **46**:466–474.
7. **García-Barreno, B., C. Palomo, C. Peñas, T. Delgado, P. Pérez-Breña, and J. A. Melero.** 1989. Marked differences in the antigenic structure of human respiratory syncytial virus F and G glycoproteins. J. Virol. **63**:925–932.
8. **Gerhard, W., J. Yewdell, M. E. Frankel, and R. G. Webster.** 1981. Antigenic structure of influenza virus hemagglutinin defined by hybridoma antibodies. Nature (London) **290**:713–717.
9. **Holland, J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol.** 1982. Rapid evolution of RNA genomes. Science **215**:1577–1585.
10. **Holland, T. C., F. L. Homa, S. D. Marlin, M. Levine, and J. Glorioso.** 1984. Herpes simplex virus type 1 glycoprotein C-negative mutants exhibit multiple phenotypes, including secretion of truncated glycoproteins. J. Virol. **52**:566–574.
11. **Jiménez, G., I. Correa, M. P. Melgosa, M. J. Bullido, and L. Enjuanes.** 1986. Critical epitopes in transmissible gastroenteritis virus neutralization. J. Virol. **60**:131–139.
12. **Lefrancois, L., and D. S. Lyles.** 1983. Antigenic determinants of vesicular stomatitis virus: analysis with antigenic variants. J. Immunol. **130**:394–398.
13. **López, J. A., M. Guillén, A. Sánchez-Fauquier, and J. A. Melero.** 1986. Antigen-binding assay to determine the specificity of monoclonal antibodies against influenza virus and mapping of epitopes. J. Virol. Methods **13**:255–264.
14. **Luria, S. E., and M. Delbrück.** 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
15. **Minor, P. D., G. C. Schild, J. Bootman, D. M. A. Evans, M. Ferguson, P. Reeve, M. Spitz, G. Stanway, A. J. Cann, R. Hauptmann, C. D. Clarke, R. C. Mountford, and J. W. Almond.** 1983. Localization and primary structure of a major antigenic site for poliovirus neutralization. Nature (London) **301**:674–679.
16. **Nakajima, S., and A. P. Kendal.** 1981. Antigenic drift in influenza A/USSR/90/77 (H1N1) variants selected in vitro with monoclonal antibodies. Virology **113**:656–662.
17. **Palese, P., and J. F. Young.** 1982. Variation of influenza A, B and C viruses. Science **215**:1468–1474.
18. **Parvin, J. D., A. Moscona, J. T. Pan, J. M. Leider, and P. Palese.** 1986. Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. J. Virol. **59**:377–383.
19. **Portner, A., K. G. Webster, and W. H. Bean.** 1980. Similar frequencies of antigenic variation in Sendai, vesicular stomatitis and influenza A viruses. Virology **104**:235–238.
20. **Reha-Krantz, L. J.** 1988. Amino acid changes coded by bacteriophage T4 DNA polymerase mutator mutants. Relating structure to function. J. Mol. Biol. **202**:711–724.
21. **Samson, A. C. R., P. H. Russell, and S. E. Hallam.** 1985. Isolation and characterization of monoclonal antibody-resistant mutants of Newcastle disease virus. J. Gen. Virol. **66**:357–361.
22. **Sherry, B., A. G. Mosser, R. J. Colonno, and R. R. Rueckert.** 1986. Use of monoclonal antibodies to identify four neutralization immunogens on a common cold picornavirus, human rhinovirus 14. J. Virol. **57**:246–257.
23. **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.
24. **Tobita, K., A. Sugiura, C. Enomoto, and M. Furuyama.** 1975. Plaque-assay and primary isolation of influenza A viruses in an established line of canine kidney cells (MDCK) in the presence of trypsin. Med. Microbiol. Immunol. **162**:9–14.
25. **Weddell, G. N., D. G. Yansura, D. J. Dowbenko, M. E. Hoatlin, M. J. Grubman, D. M. Moore, and D. G. Kleid.** 1985. Sequence variation in the gene for the immunogenic capsid protein VP1 of foot-and-mouth disease virus type A. Proc. Natl. Acad. Sci. USA **82**:2618–2622.
26. **Wiley, D. C., I. A. Wilson, and J. J. Skehel.** 1981. Structural identification of the antibody binding sites of Hong Kong influenza hemagglutinin and their involvement in antigenic variation. Nature (London) **289**:373–378.
27. **Yewdell, J., and W. Gerhard.** 1982. Delineation of four antigenic sites on a paramyxovirus glycoprotein via which monoclonal antibodies mediate distinct antiviral activities. J. Immunol. **128**:2670–2675.
28. **Yewdell, J., R. G. Webster, and W. Gerhard.** 1979. Antigenic variation in three distinct determinants of an influenza type A hemagglutinin molecule. Nature (London) **279**:246–248.