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Negative Effects of Chemical Mutagenesis on the Adaptive Behavior of Vesicular Stomatitis Virus

CAROLYN H. LEE,[1] DOROTHY L. GILBERTSON,[1] ISABEL S. NOVELLA,[1] RAMON HUERTA,[2] ESTEBAN DOMINGO,[3] AND JOHN J. HOLLAND[1]*

Department of Biology and Institute of Molecular Genetics,[1] and Institute for Nonlinear Science,[2] University of California, San Diego, La Jolla, California 92093, and Centro de Biologia Molecular "Severo Ochoa" (Consejo Superior de Investigaciones Cientificas, Universidad Autonoma de Madrid), Cantoblanco, 28049 Madrid, Spain[3]

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Changes in adaptability of vesicular stomatitis virus (VSV) upon treatment with chemical mutagens have been investigated. Results showed no improvement in virus viability or adaptability at any given level of mutagenesis. In fact, increasing inhibition of virus production and adaptability was observed with increasing levels of mutagenesis. This was true for all tested VSV variants replicating either in changing or constant host cell environments. Results also showed that mutagen-treated RNA virus populations which had undergone severe fitness declines were able to recover lost fitness completely after several large-population passages in BHK_{21} cells. The present findings illustrate the highly optimized states of RNA viruses and their potential to adapt readily. These results are significant for the possible development of specific antiviral agents designed to be mutagenic.

Within the past two decades, it has become increasingly clear that most RNA viruses are highly mutable, greatly adaptable, and capable of rapid evolution. While estimates differ because of the various methods of measurement and genome sites examined, most RNA viruses exhibit mutation rates of about 10^{-4} to 10^{-5} base misincorporations per site per round of replication (for specific mutation rates and reviews, see references 2, 3, 5, 7–9, 21–24, 29–32, and 35). The highly error-prone replication of RNA viruses is due to the absence of any efficient proofreading or repair mechanisms associated with the viral RNA polymerase (30). Hence, for RNA viruses with genome sizes of approximately 10 kb, many of the progeny will contain one or more mutations. In other words, RNA virus populations (even a population derived from a single particle) will consist of a complex mixture of related but extremely heterogeneous genomes. Eigen et al. defined these mutant swarms as quasispecies populations to represent the heterogeneity and population dynamics of most, if not all, RNA viruses (6, 7, 13, 14).

It has been suggested that RNA viruses with mutation rates in the range of 10^{-4} to 10^{-5} base misincorporations per site per round of replication replicate very close to an error threshold such that crossing it will lead to an irreversible loss of genetic information or a mutational meltdown (12, 15). The theory hypothesizes that, at polymerase error rates just below the error threshold, conditions for adaptability are most favorable because the wild type can exist in a stable form while surrounded by a maximal distribution of mutants. If the error threshold is reached (or crossed), delocalization of the mutant distribution occurs and the information of the wild type is lost (12–15). In contrast to the classical concept of evolution, the error threshold theory suggests that the target of selection is not the single best-adapted individual, but rather the whole quasispecies distribution. For example, near the error threshold, selection may favor inferior swarm mutants over a single much more fit clone by virtue of the former's superior overall mutant composition (4).

Past studies have shown that chemical mutagenesis significantly alters the phenotypes of various RNA virus populations. For instance, increasing percentages of temperature-sensitive mutants of vesicular stomatitis virus (VSV) have been shown to result from increasing levels of 5-fluorouracil (5-FU) (29). Greater-than-10-fold increases in frequencies of small-plaque mutants of Newcastle disease virus as well as of poliovirus have been observed after nitrous acid treatment (1, 17). Also reported was an increase of up to 220-fold in the frequency of large-plaque mutants of Venezuelan equine encephalitis virus following treatment with 5-azacytidine (5-AZAC) (18). Nevertheless, chemical mutagenesis has not been shown to cause the same level of effects at single genomic sites. For instance, chemical mutagenesis was unable to increase defined single-site mutation frequencies in VSV and poliovirus more than 1.1- to 2.8-fold at any level of mutagenesis or virus survival level (20).

The main purpose of this study was to investigate changes in adaptability (or fitness) of various VSV populations that have been pushed to replicate at higher mutation rates by chemical mutagenesis. The chemical mutagens used included the base analogs 5-FU and 5-AZAC and the deaminating agent nitrous acid (HNO_2). It was of particular interest to quantitate any changes in fitness of a 10-month sandfly-adapted VSV strain (MARMU[10Mo/S-A]) that normally exhibits rapid adaptation in a mammalian cell line (BHK_{21}). Changes in adaptability following chemical mutagenesis were also examined for other VSV strains previously characterized as having high fitness (MARMU-HIGH), neutral fitness (wild-type), or low fitness (B25 and B25P9).

* Corresponding author. Mailing address: Department of Biology and Institute of Molecular Genetics, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0116. Phone: (619) 534-2520. Fax: (619) 534-7108.

MATERIALS AND METHODS

Cells and culture medium. Two cell lines were employed: a mammalian cell line (BHK_{21} baby hamster kidney cells) grown at 37°C and an insect cell line (LL-5 sandfly cells) grown at 28°C. The BHK_{21} cells were originally obtained

from the American Type Culture Collection, and the LL-5 cells were derived from *Lutzomyia longipalpis* (33). BHK_{21} cells were grown as monolayer cell cultures in Eagle's minimal essential medium (MEM) containing heat-inactivated (60°C, 30 min) bovine calf serum, and LL-5 cells were grown in MM medium (28). Passaging of cells was carried out as previously described (10). BHK_{21} cell monolayers were used exclusively for all virus plaque assays. Difco protease peptone 3 was added to MEM at a final concentration of 0.06% to promote rapid growth and optimal formation of BHK_{21} cell monolayers. Protease peptone was not added to MEM used for virus replication, for virus plaque assays, or for propagation of LL-5 cells.

Virus strains. The wild-type Mudd-Summers strain of VSV Indiana serotype was originally obtained from Donald Summers and has been replicated in this laboratory exclusively on BHK_{21} cells. The clonal population of wild-type virus used was a large clonal pool stored in aliquots at −70°C. The wild type has been assigned a fitness value (W) of 1 and has been used as a neutral internal control. Monoclonal antibody (MAb)-resistant mutant (MARM) clone U (MARMU) was derived by plaque selection under I1 MAb-containing overlay medium. MARMU is neutral ($W = 1$) in competition with the wild type both in BHK_{21} cells and LL-5 cells and has been used as a genetically marked surrogate wild type. Clone MARMU-HIGH was obtained after 41 large-population passages in BHK_{21} cells at 37°C and showed a high-fitness phenotype in BHK_{21} cells ($W = 6.0 \pm 0.6$). Clone B25 was a clonal population derived from the wild type after 25 consecutive genetic bottleneck transfers of small-plaque-selected clones on BHK_{21} cells at 37°C. It exhibited a very-low-fitness phenotype in BHK_{21} cells ($W = 0.017 \pm 0.007$). B25P9 was obtained after nine large-population passages of B25 in BHK_{21} cells at 37°C, and showed a low fitness phenotype in BHK_{21} cells ($W = 0.11 \pm 0.02$). The 10-month sandfly-adapted VSV strain (MARMU [10Mo/S-A]) was obtained by infecting LL-5 cells at 28°C with neutral MARMU at high multiplicity and allowing it to replicate continuously for 10 months (28). After 10 months the virus was amplified by a single passage in fresh LL-5 sandfly cells. Since MARMU[10Mo/S-A] was found to consist of both I1 MAb-resistant and -sensitive revertant variants, neutralization of revertants was carried out by incubating 1 volume of MARMU[10Mo/S-A] and 2 volumes of I1 MAb (for 1 h at 37°C). MARMU[10Mo/S-A] was then absorbed onto an LL-5 cell monolayer (15 min at room temperature), washed several times with MM medium, and incubated for 48 h at 28°C for virus stock production. Following amplification, MARMU[10Mo/S-A] virus stocks were stored in aliquots at −85°C. MARMU [10Mo/S-A] showed an extremely low-fitness phenotype in BHK_{21} cells at 37°C ($W = 0.0000038 \pm 0.0000002$). B25, B25P9, and MARMU[10Mo/S-A] were subjected to repetitive large-population passages in BHK_{21} cells at 37°C. As previously described, the virus yield at each passage (approximately 10^{10} PFU/ml) was recovered, diluted, and transferred to uninfected cells at a multiplicity of infection of 0.1 (10).

Mutagenesis with 5-FU and 5-AZAC. BHK_{21} cell monolayers were pretreated with MEM containing various amounts of mutagens (5-FU or 5-AZAC) for 6.5 h. Pretreatment was carried out to allow equilibration of nucleoside triphosphate concentration inside the cells prior to virus infection. After pretreatment, the BHK_{21} monolayers were infected with approximately 10^7 infectious particles of VSV (multiplicity of infection of 6) diluted in MEM containing appropriate concentrations of mutagens. After time for adsorption (15 min at room temperature) and penetration of VSV into the monolayers (30 min at 37°C) had been allowed, the monolayers were washed five to six times with MEM in order to wash off nonadsorbed virus particles. MEM containing fetal calf serum with the indicated amounts of mutagens (5-FU or 5-AZAC) was then added, and the cytopathic effect was observed on the following days. When viral cytopathology was complete, the pools of viruses were titrated and their relative fitness values were determined.

Mutagenesis with nitrous acid (HNO_2). Acetate buffer (pH 4.51) was prepared from 1 M sodium acetate and 1 M acetic acid. Two volumes of virus were mixed with 1 volume of 1 M acetate buffer and 1 volume of sodium nitrite. The reaction mixture was gently and constantly stirred. Samples were taken after 3.0 and 3.5 min of treatment and diluted into alkaline MEM (pH 7.7 to 7.9) to halt mutagenesis.

Relative fitness assays and fitness vectors. Methods for fitness determinations were carried out as previously described in detail (22). Briefly, genetically marked MARM virus and wild-type virus were mixed together to give a known starting ratio, and this mixture was seeded onto a cell monolayer (BHK_{21}) to allow direct competition between the two viruses. After cytopathology was complete, the resulting mixed-virus yield was diluted 10^4-fold and seeded onto another cell monolayer to initiate the next competition passage. All competition passages for fitness determinations were carried out at 37°C and in the absence of any mutagen. At each passage, changing wild type/MARM ratios were determined by triplicate plaque assays with and without I1 MAb in the overlay medium. I1 antibody-producing mouse hybridoma cells were kindly provided by Leo Lefrancois (27), and I1 MAbs were prepared as previously described (10). The fraction of the original wild type/MARM ratio was plotted versus the competition passage number to obtain a fitness vector in which the slope represents a fitness value (W). Fitness determinations for MARMU[10Mo/S-A] and MARMU-HIGH were carried out in competition with the wild type (internal control), while fitness determinations of the wild type and B25 were carried out in competition with MARMU (the surrogate wild-type internal control).

TABLE 1. Viral yields and fitness changes of VSV strains after one passage in BHK_{21} cells in the presence of 5-FU[a]

VSV strain	5-FU concn (μg/ml)	Virus survival (% control)	Fitness
MARMU [10Mo/S-A]	None (parental)		0.0000038 ± 0.0000001
	None (control)	100	0.3782 ± 0.0002
	3	100	0.20 ± 0.04
	6	100	0.04 ± 0.01
	12.5	12.5	0.0122 ± 0.0008
	25	40	0.002224 ± 0.000008
	50	6	0.0007 ± 0.0001
	100	1	0.0019 ± 0.0002
	200	0.4	0.013 ± 0.001
	400	0.2	0.000094 ± 0.000007
Wild type	None (parental)		1.0
	None (control)	100	1.39 ± 0.09
	6	67	0.9 ± 0.1
	25	17	0.41 ± 0.04
	100	0.1	0.3 ± 0.3
MARMU-HIGH	None (parental)		6.0 ± 0.6
	None (control)	100	10.0 ± 2.0
	6	80	3.04 ± 0.07
	25	20	0.7 ± 0.1
	100	0.4	0.6 ± 0.4
B25P9	None (parental)		0.11 ± 0.02
	None (control)	100	0.224 ± 0.007
	6	20	0.0055 ± 0.0005
	25	13	0.00014 ± 0.00005
	100	0.3	0.0002 ± 0.0002

[a] One-step mutagenesis was carried out with various amounts of 5-FU as described in Materials and Methods. When cytopathology was complete, titrations and fitness assays were performed to determine changes in viral yields and fitness values.

RESULTS

An earlier study showed that continuous, persistent replication of VSV in cultured sandfly cells for 10 months had led to greatly decreased virus replicative fitness in BHK_{21} mammalian cells (28). In fact, fitness in BHK_{21} cells was observed to be over 2,000,000-fold less than the fitness observed in sandfly cells. However, following only a single passage of the sandfly-adapted VSV (or MARMU[10Mo/S-A]) in BHK_{21} cells at 37°C, fitness increased over 1,000-fold. And, after seven passages in BHK_{21} cells, fitness in BHK_{21} cells had increased nearly 10,000-fold. To examine whether the same potential for rapid adaptation was also inherent in a quasispecies population derived from only one or a few infectious particles, fitness was determined for a clonal population of MARMU[10Mo/S-A] before and after one passage in BHK_{21} cells at 37°C. Results indicated that only one round of replication of the clonal MARMU[10Mo/S-A] population in the new (BHK_{21}) environment was enough to generate a quasispecies population that had gained fitness by over 7,000-fold (data not shown). These observations underscore the potential for great adaptability of RNA viruses (even those derived directly from a clone) when introduced into new host cell environments.

To investigate the effects of mutation rate changes during rapid adaptation of RNA viruses, differences in fitness were quantitated after a single high-multiplicity passage of mutant MARMU[10Mo/S-A] in the presence of 5-FU. Table 1 shows that, at any given level, 5-FU was unable to improve either MARMU[10Mo/S-A] production or adaptability in BHK_{21} cells at 37°C. Instead, with increasing levels of mutagens, a

TABLE 2. Viral yields and fitness changes of VSV strains after one passage in BHK_{21} cells in the presence of 5-FU or HNO_2[a] or at high temperatures[b]

VSV strain	Mutagen (concn or time of treatment) or temp	Virus survival (% control)	Fitness
MARMU [10Mo/S-A]	None (parental)		0.0000038 ± 0.0000002
	None (control)	100	0.3782 ± 0.0002
	HNO_2 (3.0 min)	16	0.002 ± 0.002
	5-FU (25 μg/ml)	40	0.00222 ± 0.00001
	39°C	17.1	0.141 ± 0.03
Wild type	None (Parental)		1.0
	None (control)	100	1.4 ± 0.1
	HNO_2 (3.0 min)	14.8	0.63 ± 0.08
	5-FU (25 μg/ml)	17	0.41 ± 0.05
	40.5°C	6.6	0.6 ± 0.2
MARMU-HIGH	None (parental)		6.0 ± 0.6
	None (control)	100	10.0 ± 2.0
	HNO_2 (3.5 min)	12	2.4 ± 0.5
	5-FU (25 μg/ml)	20	0.7 ± 0.1
	40.5°C	5	4.0 ± 0.6
B25P9	None (parental)		0.11 ± 0.03
	None (control)	100	0.224 ± 0.007
	HNO_2 (3.0 min)	9.8	0.099 ± 0.03
	5-FU (25 μg/ml)	13	0.00014 ± 0.00007
	39.5°C	1	0.05 ± 0.01

[a] Mutagenesis by HNO_2 was performed as described in Materials and Methods. Titrations and fitness assays were carried out to quantitate changes in viral yields and fitness values.

[b] Single high-multiplicity passages at higher temperatures involved infection by virus of BHK_{21} cells (at a multiplicity of infection of 6) followed by viral adsorption (15 min at room temperature) and viral penetration (30 min at 37°C). Infected monolayers were then washed (five to six times) with MEM, overlaid with MEM containing fetal calf serum, and incubated at the indicated temperatures (in a constant-temperature H_2O jacket incubation chamber). When viral cytopathology was complete, titration and fitness assays (as described in Materials and Methods) were performed to determine changes in viral yields and fitness values.

tendency toward greater suppression of virus production and greater limitations of fitness gains was observed. Although virus production of MARMU[10Mo/S-A] did not drop at the lowest treatment levels of 5-FU (i.e., 3 mg/ml and 6 mg/ml), it is interesting that a limitation of fitness gains was still observed. Decreases in fitness were also observed when various amounts of 5-FU were present during high-multiplicity passages of wild-type, MARMU-HIGH, and B25P9 VSV strains replicating in constant host cell environments (BHK_{21} at 37°C) (Table 1). Results for wild-type, MARMU-HIGH, and B25P9 strains also showed tendencies toward greater fitness suppression with increasing levels of 5-FU. Hence, chemical mutagenesis (higher mutation rates) generally exerted negative effects on RNA viral viability and adaptability.

Since suppression of adaptability may be directly influenced by host cell cytotoxicity induced by chemical mutagenesis, differences in fitness were also examined for VSV treated with HNO_2 prior to infection. Again, results showed significant decreases both in survival and fitness for all VSV strains following treatment with HNO_2 (Table 2). Moreover, viral yield and fitness of HNO_2-treated MARMU-HIGH remained relatively suppressed after one further large-population passage in BHK_{21} cells at 37°C in the absence of mutagenic treatment (data not shown). Thus, the HNO_2-mutagenized MARMU-HIGH population had acquired mutations which did not allow an immediate increase in fitness.

To examine whether the decrease in viral fitness was directly related to a decrease in viral viability, fitness values for VSV were also determined after a high-multiplicity passage in BHK_{21} cells at elevated temperatures. Here, elevated temperatures (comparable to a fever) were utilized to suppress virus survival in a rather nonspecific manner. Table 2 displays fitness values for VSV passaged at temperatures ranging from 39 to 40.5°C in comparison to fitness values for VSV passaged in the presence of 5-FU. Fitness values derived from testing at high temperatures were consistently higher than fitness values derived after 5-FU treatment for all VSV strains tested. This observation held true even when the percentages of viral yields were lower for VSV passaged at higher temperatures. These results suggest that increased mutation rates (rather than reduced virus production) are the main factors in suppressing VSV adaptation by chemical mutagenesis. These findings also suggest that chemical mutagenesis works both specifically and nonspecifically to inhibit viral adaptability. It should be noted that fever temperatures have been reported to increase temperature-sensitive mutant frequencies in VSV (16). This could explain the slightly reduced adaptability which was observed for VSV strains as a result of elevated temperatures.

Differences in fitness were also determined for VSV after a single high-multiplicity passage in BHK_{21} cells in the presence of 5-AZAC. Results showed that 5-AZAC was also unable to increase the adaptability of any VSV strain employed (Fig. 1). It is important to note that the limitation of fitness gains was more dramatic for MARMU[10Mo/S-A] than were the inhibitions of parental fitness for the wild-type, MARMU-HIGH, and B25P9 strains. This may be partly explained by the very low fitness of parental MARMU[10Mo/S-A] compared to that of the other parental VSV strains. Hence, increasing mutation rates in a very poorly adapted RNA viral quasispecies population (such as MARMU[10Mo/S-A]) are more likely to lead to a greater collapse in replicative fitness due to the population's original debilitated state.

To examine whether RNA viruses had lost their potential to recover their adaptability after replicating at higher mutation rates, fitness values were quantitated for mutagenized VSV strains following several passages in untreated BHK_{21} cells.

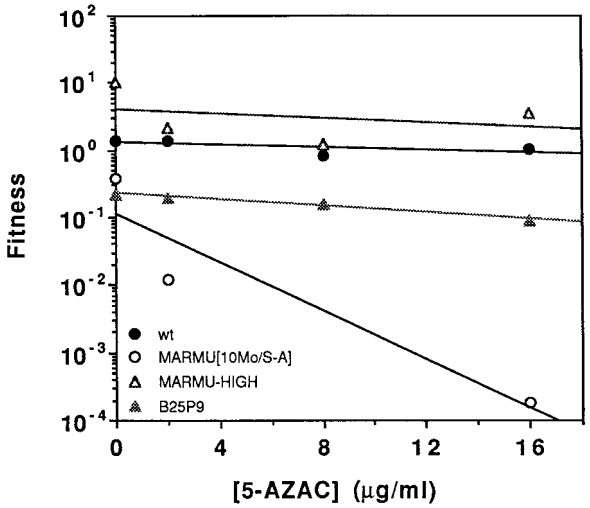

FIG. 1. Fitness changes for VSV strains after one passage in BHK_{21} cells in the presence of 5-AZAC. One-step mutagenesis was carried out with the indicated concentrations of 5-AZAC as described in Materials and Methods. When cytopathology was complete, fitness assays were performed to determine changes in fitness values. wt, wild type.

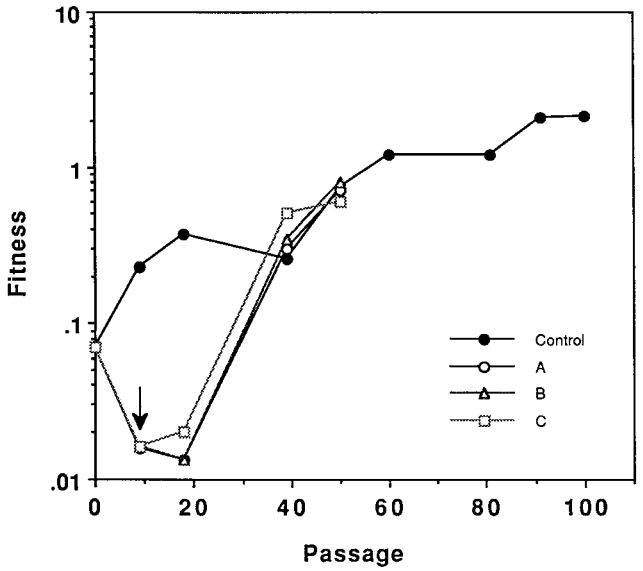

FIG. 2. Relative fitness changes of B25 during passages in BHK_{21} cells following long-term mutagenesis. B25 was subjected to nine large-population passages in BHK_{21} cells at 37°C in the presence of 25 μg of 5-FU per ml and 5 μg of 5-AZAC per ml. At each passage interval, virus was harvested and transferred at a multiplicity of infection of 0.1 to fresh BHK_{21} cells. The passage at which long-term mutagenesis was completed (passage 9) is indicated with an arrow. The kinetics of fitness recovery following mutagenesis are shown for three independent series (up to passage 50). Following mutagenesis, three independent series (A, B, and C) of 41 large-population passages were carried out in BHK_{21} cells in the absence of mutagens. Large-population passages involved diluting progeny virus from the previous passage and infecting a fresh BHK_{21} monolayer at a multiplicity of infection of 0.1. The kinetics of fitness changes show temporary decreases of fitness (for series A and B) followed by dramatic increases in fitness (for all three series). Control B25 was not subjected to any chemical mutagenesis.

Large-population passages were carried out with MARMU [10Mo/S-A] in BHK_{21} cells following a single high-multiplicity passage in the presence of 400 μg of 5-FU per ml (which greatly suppressed the usual fitness gains observed in untreated BHK_{21} cells at 37°C). Results showed that, after six passages, mutagenized MARMU[10Mo/S-A] regained fitness by nearly 9,700-fold ($W = 0.91 \pm 0.08$) such that its fitness value became comparable to that of MARMU[10Mo/S-A] after seven passages without mutagens ($W = 2.0 \pm 0.2$).

The kinetics of fitness changes of the B25 strain were also examined following long-term chemical mutagenesis with 5-FU and 5-AZAC. Three independent series of 41 large-population passages were carried out on BHK_{21} cells at 37°C following 9 passages in the presence of 25 μg of 5-FU per ml and 5 μg of 5-AZAC per ml. Fitness values were determined after long-term mutagenesis was completed (passage 9) and compared with fitness values determined for subsequent passages in untreated BHK_{21} cells. Figure 2 shows fitness changes for B25 before and after the nine passages in the presence of chemical mutagens. In agreement with earlier results, suppression of adaptability was observed following mutagen treatment (passage 9). Unexpectedly, further (but temporary) drops in fitness were observed for series A and B after nine large-population passages of mutagenized B25 in untreated BHK_{21} cells (passage 18). However, this was followed by dramatic increases in fitness gains (for all three series) during subsequent passages in the absence of mutagens. After 30 passages in untreated BHK_{21} cells (passage 39), the kinetics of fitness gains for mutagenized B25 (series A, B, and C) and control B25 became comparable. The present findings suggest that RNA viruses inevitably recover their lost adaptability once they are no longer subjected to chemical mutagenesis (or higher mutation rates) as long as there are surviving mutants that are able to replicate, mutate, and adapt.

DISCUSSION

The fitness gain results for VSV upon treatment with chemical mutagens showed no improvement in viral production or adaptability at any given level of mutagenesis. On the contrary, greater inhibition of viral yield and adaptability was generally observed with increasing levels of chemical mutagenesis. Because mutation frequencies (at defined sites) do not increase more than threefold for the surviving VSV mutants (20), our results strongly suggest that VSV replicates with a fidelity which is highly optimized for adaptation and evolution. In addition, the significant limitations of MARMU[10Mo/S-A] fitness gains observed after 5-FU or 5-AZAC treatment and the significant decreases of parental B25P9 fitness observed after 5-FU treatment support the prediction that viruses of lower fitness may replicate closer to error catastrophe because of the decreased superiority of the master sequence over the mutant spectrum (12–15).

It has been suggested that the manipulation of RNA virus mutation rates may prove to be effective in combating viral infections (11, 25). For instance, drugs that increase mutation rates may cause RNA viral quasispecies to lose meaningful genetic information, while drugs that decrease mutation rates may cause a reduction of viral diversity and thereby minimize the generation of resistant mutants. For instance, iododeoxyuridine has been widely used in treating herpes simplex virus infections. Upon phosphorylation by viral kinases, iododeoxyuridine has been observed to exhibit mutagenic effects in virus-infected cells by increasing base misincorporations (19), suggesting that iododeoxyuridine pushes the herpes simplex virus to replicate closer to and beyond its error threshold. A recent study showed that human immunodeficiency virus (HIV)-infected patients receiving 3TC monotherapy (for more than 12 weeks) exhibited not only high-level resistance to 3TC but also stabilized titers of HIV-neutralizing antibodies (in contrast to rapid declines in patients treated with AZT). The methionine 184-to-valine substitution conferring 3TC resistance was also reported to cause increased fidelity to viral reverse transcriptase (34).

According to the error threshold concept of Eigen et al. (12–15), it is expected that chemical mutagenesis could push quasispecies populations near the error threshold. It is conceivable that the development of virus-specific mutagenic drugs (in addition to other antiviral agents) could reduce viral loads if the error threshold could actually be crossed and could assist the immune response in clearing viral infections. Greater knowledge of the structures and functions of viral proteins (such as the viral polymerase and kinases) will be crucial in designing effective virus-specific mutagenic drugs. In addition, since there are differences in the modes of actions of various chemical mutagens on different types and strains of viruses, investigations of the physiological effects and molecular mechanisms of chemical mutagenesis on host cells and viruses will be required.

It is important to appreciate the extreme variation and extraordinary potential of RNA viruses to undergo rapid evolution when attempting to design measures to control and eradicate RNA viral quasispecies. Our findings show that RNA viruses are able to recover lost fitness after only several population passages following severe chemical mutagenesis. This was true for both short-term (MARMU[10Mo/S-A]) and long-

term (B25) chemical mutagenesis. Further studies on the quasispecies nature and population dynamics of RNA viruses will become increasingly important not only for the understanding of viral evolution and pathogenesis but also for the development of effective prophylaxis and treatment.

Finally, it should be noted that the mutation rates per genome for DNA-based organisms are only about 300-fold higher than those for RNA viruses because their mutation rates per base pair are inversely proportional to genome size, and they have evolved a rather constant mutation rate per genome of $\approx$0.003 (discussed by Drake in reference 9 and references therein). Therefore, it is not surprising that spontaneous mutation rates alone may often be sufficient to reduce fitness in higher DNA-based organisms (26), nor that additional mutagenesis might often further reduce fitness. Regardless, our results clearly demonstrate that mutagenesis of RNA viruses exerts deleterious effects on fitness.

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