

# Molecular indetermination in the transition to error catastrophe: Systematic elimination of lymphocytic choriomeningitis virus through mutagenesis does not correlate linearly with large increases in mutant spectrum complexity

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**Studies with several RNA viruses have shown that enhanced mutagenesis resulted in decreases of infectivity or virus extinction, as predicted from virus entry into error catastrophe. Here we report that lymphocytic choriomeningitis virus, the prototype arenavirus, is extremely susceptible to extinction mutagenesis by the base analog 5-fluorouracil. Virus elimination was preceded by increases in complexity of the mutant spectra of treated populations. However, careful molecular comparison of the mutant spectra of several genomic segments suggests that the largest increases in mutation frequency do not predict virus extinction. Highly mutated viral genomes have escaped detection presumably because lymphocytic choriomeningitis virus replicates at or near the error threshold, and genomes in the transition toward error catastrophe may have an extremely short half-life and escape detection with state-of-the-art cloning and sequencing technologies.**

**R**NA viruses replicate with an extremely high mutation rate ( $10^{-3}$  to  $10^{-5}$  misincorporations per nucleotide copied) because of the low fidelity of RNA polymerases and reverse transcriptases, which lack proofreading repair activities (1–3). Coupled to recombination, high yields and short replication cycles, large varieties of related mutant genomes, defined as viral quasispecies, represent the genomic structure of RNA virus populations (4). The theoretical quasispecies is a steady-state organized distribution of error copies of the most fit (or master sequence) in a particular environment.

The fast generation of a large repertoire of mutant virus genomes has been interpreted as the molecular mechanism underlying the rapid evolution of RNA viruses during *in vivo* infections (5–9) and has important clinical implications. For example, it has been shown that genomes resistant to chemotherapeutic agents can already be detected in an HIV-infected person before any exposure to these agents (10). Computer simulations of the behavior of quasispecies predict that during genome replication, the mutation rate can increase only up to a threshold value without compromising the production of infectious genomes. Crossing this threshold will lead to the complete loss (or “melting”) of the genetic information. This is called error catastrophe of replication, and its induction would lead to an elimination of the infection; i.e., any viral genomes produced would contain so many misincorporations that their genomes could not replicate further (11, 12). The transition into error catastrophe would convert a productive infection into an abortive one (13).

Considerable evidence suggests that riboviruses exhibit a genome mutation rate that is very close to the predicted threshold for entry into error catastrophe of replication. Consistent with this hypothesis, exposure of RNA viruses to mutagenic agents leads frequently to abortive infections. Thus, when examined, the mutation fre-

quency of poliovirus and vesicular stomatitis virus could not be increased at defined loci by more than 3-fold by using chemical mutagenesis [e.g., 5-fluorouracil (FU), 5-azacytidine (AZC), ethylmethanesulfonate, or nitrous acid (14)]. In similar experiments the retrovirus mutation rate was increased by 13-fold after treatment with the nucleoside analog AZC (15). In another study 3'-azido-3'-deoxythymidine (AZT) was shown to increase the rate of HIV-1 mutation by a factor of 7 in a single round of replication (16).

Further evidence in support of mutagenesis-induced virus elimination has been obtained with HIV-1 by using deoxynucleoside analogs to increase viral genome mutagenesis (17). Those authors coined the term “lethal mutagenesis” to describe mutagen-induced loss of viral infectivity and suggested this could provide a new approach to treating HIV-1 and other RNA virus infections. The same authors also proposed to induce lethal mutagenesis through the incorporation of mutagenic ribonucleosides into the HIV-1 genome by the host cell RNA polymerase, rather than by the viral reverse transcriptase to avoid resistant viruses (18).

Recently, we passaged foot-and-mouth disease virus (FMDV) in the presence of FU and AZC and characterized the viral genomes through intensive sequencing analysis. Occasionally, but not systematically, FMDV was driven into extinction concomitantly with an increase in mutation frequency and mutant complexity (19). In a follow-up study we demonstrated that reducing viral loads and viral fitness systematic extinctions of FMDV were achieved accompanied by an increase in mutant spectrum complexity (20).

In a more recent study Crotty *et al.* (21) suggested that ribavirin's antiviral activity may be exerted through lethal mutagenesis. They described that ribavirin caused a large (99.3%) loss of viral genome infectivity, concomitantly with only a 9.7-fold increase in mutagenesis, and suggested that ribavirin forced poliovirus into error catastrophe (22).

Lymphocytic choriomeningitis virus (LCMV), a noncytopathic arenavirus in cell culture and a natural pathogen of wild mice, has been an important model to study persistent infections and to define the role of the immune system in viral pathogenesis (23–29). LCMV has a single-stranded RNA genome of negative polarity composed of a large (L) and a small (S) RNA segment (30). The complete nucleotide sequence reveals an ambisense coding strategy on both RNA segments encoding two proteins in opposite orientations: a nucleocapsid protein (NP) and an envelope glycoprotein

Abbreviations: 5-FU, 5-fluorouracil; AZC, 5-azacytidine; FMDV, foot-and-mouth disease virus; LCMV, lymphocytic choriomeningitis virus; GP, glycoprotein; NP, nucleocapsid protein; pfu, plaque-forming unit.

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(GP) on the small RNA segment (31, 32), and an RNA polymerase and a small zinc-finger protein (Z) on the large RNA segment (30, 33). LCMV is an excellent model to study the generation and selection of viral variants in cultured cells and *in vivo*. The *in vivo* biological significance of LCMV variants has been a matter of study, but the complexity of LCMV quasispecies has not been examined in detail (34–36).

Serial passages of LCMV quasispecies in the presence of increasing concentrations of the nucleoside analogs AZC and FU demonstrated that systematic elimination of the virus can be obtained with FU in a dose-dependent fashion. Despite achieving systematic elimination of LCMV infections *in vitro*, and up to 15-fold increases in mutation frequency during viral replication in the presence of mutagens, the systematic virus elimination did not correlate with the large increases in the number of misincorporations per nucleotide, but did correlate with our inability to amplify any viral sequences.

## Materials and Methods

**Cells and Virus.** Baby hamster kidney cells (BHK-21) were grown as described (19). Vero p68 cells were maintained in DMEM supplemented with 5% FCS and 2% glutamine. LCMV ARM 53 b is a triple plaque-purified clone from ARM CA, passaged four times in BHK-21 cells. A stock virus (p0) was prepared by infecting BHK-21 monolayers ( $1 \times 10^7$  cells) with 0.01 plaque-forming unit (pfu) of LCMV per cell.

**Virus Infections.** Conditions for a standard infection were selected to obtain high virus titers ( $\geq 10^8$  pfu/ml). Semiconfluent [ $2 \times 10^6$  cells in 100-mm diameter dishes (Greiner, Nürtingen, Germany)] monolayers of BHK-21 were infected with 0.01 pfu per cell (0.001 pfu per cell for the low viral load infections in serial passages in the presence of AZC) in 5 ml of DMEM supplemented with 10% FCS, 10% tryptose phosphate broth, 2% L-glutamine, 0.52% glucose, and 50  $\mu$ g/ml gentamicin. Viral supernatants were harvested 48 h postinfection, clarified by centrifugation at 2,500 rpm for 30 min at 4°C, and stored at –80°C.

For various preextinction virus populations (virus infectivity  $\leq 10^3$  pfu/ml) (passages 1 and 2 of LCMV grown in 100  $\mu$ g/ml of FU, and passage 9 of virus grown in 25  $\mu$ g/ml of FU) 15 flasks of semiconfluent BHK-21 monolayers ( $1.6 \times 10^7$  cells per 182-cm<sup>2</sup> flask) were infected and incubated for 48 h in 10 ml of infection medium per flask. Virus sequences could be only amplified from passage 1 in 100  $\mu$ g/ml. No specific LCMV sequences could be amplified from passage 2 in 100  $\mu$ g/ml or from passage 9 in 25  $\mu$ g/ml. Virus infectivity was determined by plaque assay on Vero cell monolayers as described (37). Values shown are the mean of three titrations.

**Drug Treatment.** Preparation of AZC and FU stock solutions, determination of BHK-21 cell viability, and procedures for infections in the presence of mutagen nucleoside analogs have been described (19).

**RNA Extraction, Reverse Transcription–PCR, Nucleotide Sequencing, and Calculation of Mutant Spectrum Complexity.** RNA was extracted with Trizol (Sigma) from supernatants of infection following the manufacturer's protocol. Virus suspensions with low infectivity ( $\leq 10^3$  pfu/ml) were concentrated 20-fold by ultrafiltration (Centricon Plus-20 Biomax-100, Millipore) before RNA extraction, and RNA was resuspended in 1/10 the volume of control samples.

RNAs were amplified in triplicate by reverse transcription–PCR by using avian myeloblastosis virus reverse transcriptase and *Pfu* DNA polymerase (both from Promega). As a control to confirm that an excess of template was being amplified, a 1/10 dilution of the RNA was processed in parallel. cDNAs were purified with a Wizard PCR purification kit (Promega) and cloned into pGEM-T Easy Vector (Promega) that was used to transform *Escherichia coli*

DH5 $\alpha$  to obtain molecular clones. Purified cDNA and molecular clones were subjected to cycle sequencing with Big Dye chemistry (Applied Biosystems; Perkin–Elmer), and the products obtained were analyzed with an ABI 377 automated sequencer (Perkin–Elmer).

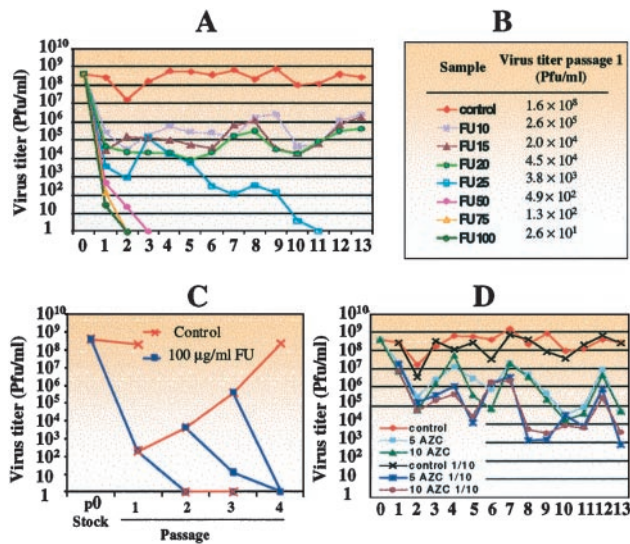
Three LCMV genomic regions were amplified by reverse transcription–PCR and sequenced by using the following oligonucleotide primers: in the small genomic RNA the GP genomic region, using primers GP247 (forward 5'-GTG GCA TGT ACG GTC TTA AGG-3') and GP759 (reverse 5'-GGT ATT GGT AAC TCG TCT GGC-3'); and the NP region with primers NP2223 (forward 5'-GCA TTG TCT GGC TGT AGC TTA-3') and NP2743 (reverse 5'-CAA TGA CGT TGT ACA AGC GC-3'). In the large RNA, the polymerase region was amplified with primers L3654 (forward 5'-AGT TTA AGA ACC CTT CCC GC-3') and L4233 (reverse 5'-TGT TGA GGG TTC CAC AGA GC-3'). All of the nucleotide positions are given in the viral (genomic) sense. Sequences were retrieved from GenBank although a reconstruction of large genomic RNA was performed by using the data from the two published sequences (accession nos. M27693 and J04331).

Total cDNA (for consensus sequences) and molecular clones obtained from passages 1 and 9 of control, FU, and AZC treatments of the virus as well as p0 (our initial stock) were sequenced. The consensus sequence of p0 was compared with the published sequence and four nucleotide substitutions in the GP gene (C467  $\rightarrow$  T, G603  $\rightarrow$  A, C606  $\rightarrow$  G, and G607  $\rightarrow$  C) were found that accounted for two amino acid changes (D176  $\rightarrow$  N and R177  $\rightarrow$  A) of the GP1 protein, whereas one nucleotide substitution was found in the NP gene at position A2290  $\rightarrow$  G. In the mutagenized populations only one nucleotide substitution was found in the consensus sequence of passage 9 of 5  $\mu$ g/ml AZC. All of our calculations are based on mutations found by alignment of consensus sequences compared with that of p0, and, within each quasispecies analyzed, only mutations not present in the corresponding consensus sequence were counted. An average of 15 clones per sample were sequenced (7,000–11,000 nt per sample in each genomic region were screened for substitutions, deletions, or insertions). Sequences were compared by using BLAST TWO sequences (<http://www.ncbi.nlm.nih.gov/gorf/bl2.html>) and SEWER (<http://iubio.bio.indiana.edu/webapps/SeWeR/>).

The complexity of the quasispecies was analyzed by means of two parameters: (i) mutation frequency and (ii) Shannon entropy. The first represents the number of mutations per nucleotide, and the second is a measure of the number of different molecules in the mutant spectrum of the quasispecies. To calculate the mutation frequency of each quasispecies, repeated mutations were counted only once. Shannon entropy is calculated with the formula:  $-\sum_i [p_i \times \ln p_i / \ln N]$  in which  $p_i$  is the frequency of each sequence in the quasispecies and  $N$  is the total number of sequences compared (38). Shannon entropy value of 0 indicates that all of the sequences are identical whereas a value of 1 indicates that every molecule differs from the others in its nucleotide sequence.

## Results

**Genomic Viral Quasispecies Structure of LCMV: Experiments with FU.** We characterized the genomic structure of LCMV during serial passages in the presence of the mutagenic base analogs FU or AZC. We measured and compared viral infectivity and mutation frequencies of mutagenized and nonmutagenized LCMV populations. The behavior of LCMV quasispecies in serial passages in the presence of increasing concentrations of FU is illustrated in Fig. 1A. Infectivity values after a single passage (given in Fig. 1B) show the dose–response effect of FU on LCMV replication. At low concentrations (10, 15, 20  $\mu$ g/ml) LCMV replicates at a lower infectivity ( $\approx 3$ –4 log decrease) but it is not lost, even after 13 passages. An increase in FU to 25  $\mu$ g/ml forces LCMV to move toward extinction in 11 passages. At 50, 75, and 100  $\mu$ g/ml of FU, we uncovered a



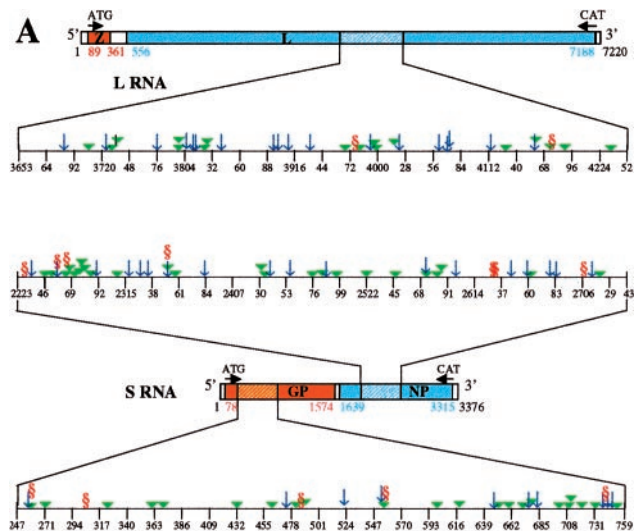
**Fig. 1.** Evolution of LCMV during serial passages in the presence of FU (A–C) and AZC (D). Virus serial passages were obtained by infection of BHK-21 monolayers with 0.01 pfu per cell of LCMV (p0, stock) in the absence (control) or presence of the indicated concentrations of nucleoside analogs (FU and AZC in  $\mu\text{g/ml}$ ). Each supernatant was assayed for virus infectivity and used as inoculum for the following passage. (A) LCMV dose–response to FU. (B) Virus infectivity titers of the first passage in the presence of increasing concentrations of FU; each value is the mean of three separate experiments. (C) Recovery of LCMV infectivity after mutagenesis with 100  $\mu\text{g/ml}$  FU. Blue lines indicate virus passages in medium containing 100  $\mu\text{g/ml}$  FU; red lines represent incubations without mutagen (control). (D) Evolution of LCMV in the presence of AZC. Multiplicity of infection in series 1/10 of control, 5  $\mu\text{g/ml}$  AZC, and 10  $\mu\text{g/ml}$  AZC was 0.001 pfu/cell per passage.

drastic effect: virus infectivity could not be detected after either two or three passages.

At the dose of 50, 75, and 100  $\mu\text{g/ml}$  of FU, virus extinction was achieved systematically in just two passages in 3–5 separate experiments. However, total recovery of the infectivity of LCMV after a  $\geq 6$  log reduction in viral titer in 100  $\mu\text{g/ml}$  of FU was obtained in three further serial passages in the absence of mutagen (Fig. 1C). At any recovery passage, the virus could still be eliminated systematically by further addition of 100  $\mu\text{g/ml}$  of FU in 1–2 further passages. Once the virus infectivity is effectively undetectable, even serial blind passages did not allow viral titers to re-emerge, confirming complete elimination of LCMV infectivity.

**Studies with AZC.** Evolution of LCMV during serial passages in the presence of high doses of AZC showed that at any of the two concentrations tested virus elimination was not observed in at least 13 passages (Fig. 1D). The highest dose of AZC not to affect the survival of BHK cells was 10  $\mu\text{g/ml}$  (19). Previously, we showed that for FMDV passaged in the presence of AZC the lowering of the viral load enhanced the frequency of extinction events (19). To test whether reducing viral load in the presence of AZC could enhance the elimination of LCMV, we serially passaged the virus at a 10-fold dilution of the multiplicity of infection of control and AZC-treated series. In these experiments a 10-fold reduction of the multiplicity of infection did not lead to systematic viral extinction over 13 passages.

**Uneven Distribution of Mutations in the LCMV Genome.** LCMV has two genomic single-stranded RNAs, large and small. Each one encodes for two ORFs arranged in an ambisense orientation (Fig. 2A). Large RNA (7.2 kb) harbors the genes for the polymerase or L protein and also a small zinc finger protein. The small RNA (3.3 kb) encodes for the GP precursor of GP1 and GP2 and NP. We have sequenced three genomic regions of molecular clones of LCMV



**Table 2: Distribution of mutations by genomic regions in LCMV control and nucleoside analog-treated populations.**

	L		GP		NP	
	n	%	n	%	n	%
Control	2	14	5	36	7	50
FU	18	39	10	22	18	39
AZC	17	27	22	35	24	38

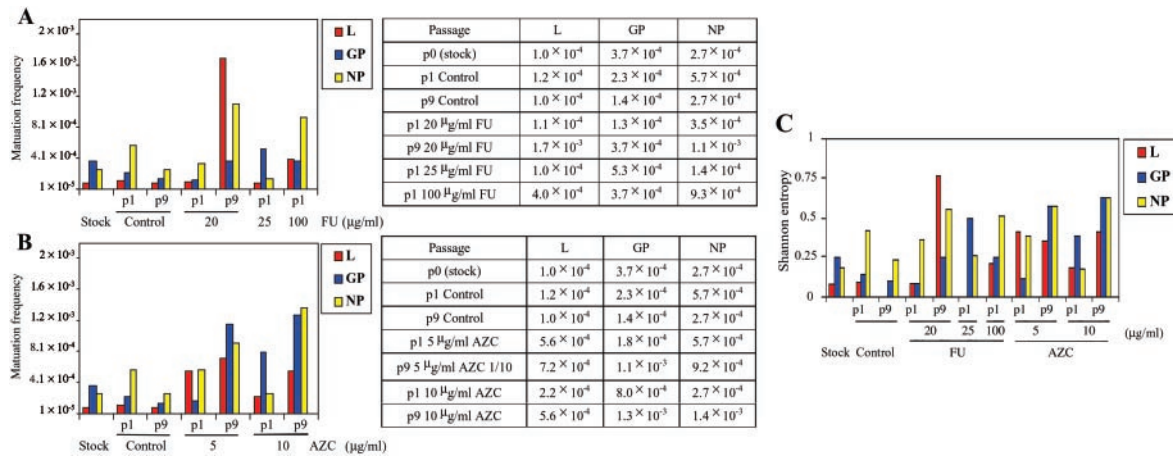
L (polymerase), GP (glycoprotein) and NP (nucleoprotein).

**Fig. 2.** (A) Schematic diagram showing the positions of mutations found in the genomic regions of control and mutagenized LCMV quaspecies. Each single-stranded genomic segment encodes for two proteins in an ambisense orientation. Positions of start and end codons for each ORF as well as mutations within the L (polymerase), GP, and NP genes are given in the genomic sense. Mutations (base substitutions, deletions, or insertions) found in the analyzed virus populations (see Table 1) are represented as § for untreated virus (control), ↓ for FU-treated virus, and ▼ for AZC-treated virus, irrespective of the passage at which they were found. The hashed areas represent the sequenced genomic regions. (B) Distribution of mutations by genomic regions in LCMV control and nucleoside analog-treated populations. Numbers (n) of mutations and percentages of the total for each treatment are given.

populations passaged in the presence or absence of FU and AZC and compared with the consensus (average) sequence. The locations of the mutations found shown in Fig. 2A indicate that there are great differences in the number of mutations detected between regions. In naive LCMV populations (see Fig. 2B) mutations in the NP and GP regions are 3.5-fold and 2.5-fold more abundant than in the L (polymerase) region, indicating that the polymerase is a conserved region and suggesting that mutations in the L region could be less tolerated than in the other two. Although the overall frequency of mutations was augmented in all regions of the LCMV genome, both mutagens induced a proportionally higher increase of mutations in the L region. Strikingly, of the two mutagens tested, AZC was able to induce the highest number of mutations in all regions analyzed. Nevertheless, AZC-treated virus was not eliminated even when viral load was decreased by 10-fold (see Fig. 1D).

**Molecular Landscape of LCMV's Genomes During Entry into Error Catastrophe.** To measure the genetic complexity of the viral quaspecies in mutagenized LCMV populations we calculated the mutation frequencies and Shannon entropy values for p0 (stock) virus and passages 1 and 9 for LCMV control and mutagen-treated populations (Fig. 3).

In the absence of published data on mutation frequencies for untreated LCMV populations we did calculate those first. In control viruses, the values for any of the three regions analyzed ranged between  $1.0 \times 10^{-4}$  and  $5.7 \times 10^{-4}$  substitutions per



**Fig. 3.** Genetic heterogeneity of LCMV quasispecies replicated in the presence of mutagenic nucleoside analogs. Mutation frequencies (A and B) and Shannon entropy (C) were calculated for the indicated LCMV passages in the absence (control) or presence of FU (A and C) and AZC (B and C). The mutation frequencies (A, tables) were calculated by dividing the number of mutations found in the viral population (and not present in its consensus sequence) by the total number of nucleotides sequenced (7,000–11,000 nt). The normalized Shannon entropy (B) was calculated as  $-\sum_i (p_i \times \ln p_i) / \ln M$  in which  $p_i$  is the frequency of each sequence in the quasispecies and  $M$  is the total number of sequences compared. Genomic regions analyzed were: L (polymerase), NP, and GP. Passage 9 in the presence of 5 µg/ml AZC is from the 1/10 dilution series.

nucleotide. After nine passages in the absence of drugs, mutation frequencies were between  $1.0 \times 10^{-4}$  and  $2.7 \times 10^{-4}$  substitutions per nucleotide. The highest mutation frequency detected corresponded to the NP region of passage 1 and the lowest was for L in agreement with previous observations (Table 1) that NP was the most variable and L the most conserved region (34–36). Shannon entropy values correlated with the detected mutation frequencies and were usually less than 0.25 except for the NP region of passage 1, which showed a higher mutant complexity (Fig. 3).

When LCMV populations were serially passaged in FU we uncovered large variations in mutation frequencies between regions. Unexpectedly, passage 9 of virus treated with 20 µg/ml FU showed a 16.8-fold increment in mutation frequency in the L region whereas in the GP region it was just 2.6-fold and 4.1-fold in the NP region. These results demonstrate, first, that it is of vital importance to analyze different areas in the genomic RNA because mutation frequencies can be region specific. If we just take into account the GP region we will assume that FU can increase the mutation frequency only 2.6-fold instead of nearly 17-fold. Second, high increments in mutation frequency in a conserved genomic region encoding for the polymerase did not abolish replication of the virus (see Fig. 1A). Intriguingly, the LCMV quasispecies is accepting at least 17-fold more mutations than the control in this particular region. The infectious titer of this virus population is over  $10^4$  pfu/ml, 3–4 logs less than the control viral quasispecies, and did not become extinct for at least four additional passages in the presence of FU (Fig. 1A).

We were only able to analyze molecular clones from one pre-extinction population, the one treated with 100 µg/ml FU. Just one passage away from extinction the mutation frequency of the polymerase increased 3.4-fold compared with the 1.6-fold found for the GP and NP regions.

For AZC-treated populations, values of mutation frequencies were similar for the three genomic regions and showed an increment of 5.1- to 9-fold compared with the control after nine passages for any of the concentrations tested. The L region was the less mutated of the three, suggesting that the type of mutagen used affects evolution of the virus. For all of the mutagen-treated populations Shannon entropies were higher than the controls and correlated with the variations of mutation frequencies, the maximum observed for the population treated for nine passages with 20 µg/ml FU.

Importantly, in two pre-extinction populations (100 µg/ml of FU on passage 2 and 25 µg/ml on passage 9) specific LCMV sequences

could not be amplified. This failure to amplify LCMV-specific sequences could not be overcome, even after large scale-up production of potentially infectious virus. In these populations mutation frequencies possibly became so high as to include mutations in the primer binding sites, and highly mutated sequences were highly unstable, given the proximity of RNA virus replication to the error threshold (4–6, 11–14, 39–43). New methods are needed to attempt to detect and analyze highly mutated, minority LCMV genomes that may transiently be produced during the transition toward error catastrophe.

**Mutations Induced by FU and AZC in LCMV Populations.** The types of mutation that were found in control, FU-treated, and AZC-treated populations are shown in Table 1. The most frequent types of mutation induced by FU were transitions  $U \rightarrow C$  and  $A \rightarrow G$  followed by  $C \rightarrow U$ , which are expected because of the chemical structure of the FU and the effect that this analog has on the cellular levels of dNTPs. In the AZC-treated populations, transversions  $C \rightarrow G$  were the most frequent followed by  $C \rightarrow A$ ,  $G \rightarrow C$ , and  $G \rightarrow U$ , in agreement with previous evidence (39). It is remarkable that in just one passage in the presence of 100 µg/ml of FU we have detected 11 transitions caused by this mutagen but only two in the sample treated with 20 µg/ml, which can account for the deleterious effect of 100 µg/ml FU on the virus population. Taken together, these results confirm that these base analogs are introducing mutations in the viral RNA. However, the high mutation frequency and Shannon entropy values for the polymerase (L) genomic region of the quasispecies of passage 9 in 20 µg/ml FU did not correlate systematically and linearly with elimination of the quasispecies.

## Discussion

Lethal mutagenesis induced by treatment with nucleoside analogs has been suggested as a novel way of treating HIV infections (17). The basis of this new antiviral therapy resides in the fact that increasing the already high mutation rates of RNA viruses would lead to the loss of the genetic message (melting of information) of the virus. Forcing the virus to cross the error catastrophe threshold would convert a productive into an abortive infection because of an excess of mutations. This is a transition predicted by quasispecies theory (4, 11–13). Base or nucleoside analogs, in particular FU and AZC, have been used to induce mutagenesis in several RNA viruses (14, 19, 44). Several studies have shown that nucleoside analog-induced loss of viral infectivity paralleled moderate increases in

**Table 1. Types of mutations found in LCMV populations subjected to chemical mutagenesis with 5-FU and AZC**

Mutation	No. mutagen			5-FU				AZC			
	p0	p1	p9	20 µg/ml		25	100	5 µg/ml		10 µg/ml	
				p1	p9	p1	p1	p1	p9	p1	p9
A → C	0	0	0	0	0	0	0	0	0	0	0
A → G	1	1	0	0	13	0	2	1	0	0	1
A → U	0	1	0	0	0	0	0	0	1	0	0
G → A	1	0	0	1	0	1	0	0	1	0	0
G → C	0	0	0	0	0	0	0	2	5	1	4
G → U	1	1	1	0	0	0	0	0	1	1	4
C → A	0	0	0	0	0	1	0	1	5	3	5
C → G	0	0	0	0	0	0	0	1	9	2	9
C → U	0	2	0	0	0	0	4	2	0	0	1
U → A	0	0	0	1	0	0	0	0	0	0	0
U → C	2	2	1	2	13	2	5	2	1	1	2
U → G	1	0	1	0	0	0	0	0	0	0	0
Deletions	0	0	0	0	1	1	1	0	1	0	0
Insertions	0	0	0	0	0	0	0	1	0	0	0
<b>Total</b>	6	7	3	4	27	5	12	10	24	8	26
<b>No. of mutations per nt* (× 10<sup>-3</sup>)</b>	0.24	0.2	0.13	0.16	1.1	0.2	0.52	0.45	0.91	0.39	1

Alternating shading is used to group passage numbers in the presence of individual mutagens' concentration.  
 \*Mutations found in total (regardless of the genomic region) divided by the total number of nucleotides sequenced in each passage.

mutation frequency during single infection events (21, 22) or after serial passages (17–20). Thus, it has been suggested that RNA viruses replicate near the error catastrophe threshold, and more recent experiments have been taken as evidence that this threshold is effectively crossed by treating poliovirus with ribavirin (22). However, although in these experiments ribavirin eliminated 99% of poliovirus genome infectivity, approximately 10<sup>7</sup> pfu were still detected, which is higher than titers found for many other viruses even in the absence of any treatment.

We have now explored extinction mutagenesis of the prototype arenavirus, LCMV. To provide detailed evidence of the molecular landscape during the transition of RNA virus replication into error catastrophe we have analyzed LCMV viral quasispecies passaged in the presence of the nucleotide analogs AZC and FU. Serial passages in the presence of increasing doses of FU showed very different outcomes of LCMV quasispecies evolution under the selective pressure of the mutagen. Within a narrow range of mutagen concentration we observed the systematic extinction of virus passaged in concentrations of 50 µg/ml FU or above, progressive loss of infectivity toward extinction after 11 passages in 25 µg/ml FU, and a decrease in replication ability of the virus passaged in 10–20 µg/ml FU. When grown in the presence of AZC, virus extinction was not observed either in 13 passages at any concentration of AZC or by decreasing the virus load 10-fold. This finding is in contrast with previous results that documented that FMDV can occasionally be driven to extinction by AZC (19). Higher concentrations of AZC could not be used because they induced unacceptably high toxicity in BHK cells.

Nucleotide sequence analyses of the three genomic regions of virus quasispecies treated in the presence or absence of mutagen showed no correlation with virus elimination. Increases in mutation frequency in the regions analyzed preceded viral extinction, and our results do not exclude that in other genomic regions mutations accumulated in a way as to direct viral extinction. The polymerase

gene of virus passaged nine times in 20 µg/ml of FU, a virus that was not eliminated by mutagen treatment, showed the highest mutation frequency, 15-fold more than passage 9 of control virus. Conversely, the virus treated with 100 µg/ml of FU, a pre-extinction population, only showed an increment of 3.4-fold for the same region compared with the control. Treatment of LCMV with 5 µg/ml or 10 µg/ml AZC was not able to drive the virus to extinction, although very high and comparable numbers of nucleotide misincorporations were detected.

Error catastrophe (4, 13, 45, 46) is predicted to involve accumulation of errors in successive replication rounds until the genomic information is entirely lost (4, 46). In this article, we show evidence that LCMV treated with 100 µg/ml FU can be systematically driven into error catastrophe. After one passage in the presence of the mutagen the virus infectivity was seriously compromised, and an additional blind passage in the presence of the drug was necessary to effectively eliminate LCMV infectivity. However, a second passage in the absence of mutagen allowed the virus to regain full infectivity as predicted by viral quasispecies theory. Although the molecular basis of entry into error catastrophe of replication can be found only in the genetic material of the virus, failure to amplify highly mutated sequences precludes such direct demonstration. Thus, our data strongly suggest that the relatively lower increases in viral mutation frequencies that can be obtained experimentally (e.g., up to 17-fold) may reflect only those genomes that can still be amplified.

Previous studies on mutagenesis of HIV and poliovirus with nucleoside base analogs calculated mutation frequencies focusing their analyses on just one genomic region. Our results have shown that mutations in the LCMV populations were not evenly distributed throughout the virus genome. We observed areas where both amounts and positions of mutations introduced by FU and AZC differ from one gene to the other. Thus, mutation frequencies from just one genomic region might lead to wrong conclusions about the

molecular scenario during mutagenesis of LCMV, and ideally the whole genome ought to be sequenced.

Differences in mutation frequencies among several genomic regions subjected to enhanced mutagenesis may be determined in part by differences in the tolerance of these individual genomic regions to additional mutations, which in turn may depend on the particular mutagenic agent used. Some of the mutated genomes analyzed might have already accumulated extremely debilitating mutations that could compromise their viability. Further studies are needed to clarify these possibilities.

In Table 1 we show the types of mutations found in the three genomic regions of each sample analyzed. Considering just the transitions induced by FU the number of mutations in the FU-treated populations would be two transitions for passage 1 of 20  $\mu\text{g/ml}$  FU and passage 1 of 25  $\mu\text{g/ml}$  FU, 26 transitions for the passage 9 of 20  $\mu\text{g/ml}$  FU, and 11 transitions for passage 1 of 100  $\mu\text{g/ml}$  FU. This finding suggests that the dose-response effect we had detected in the loss of infectivity caused by FU treatment is also found in the number of mutations typically induced by this base analog in the viral genome. This could explain that the virus treated with 100  $\mu\text{g/ml}$  is driven to extinction in just two passages because the mutations introduced in the genome are proportional to the amount of mutagen available in the cell.

The most abundant types of mutation found in the AZC-treated population were transversions  $C \rightarrow G$ ,  $C \rightarrow A$ ,  $G \rightarrow C$ , and  $G \rightarrow U$ , which are the reported mutations induced by AZC (19). The fact that in passage 9 of 10  $\mu\text{g/ml}$  AZC treatment four times more  $G \rightarrow U$  mutations were found could be caused by an excess of AZC. Thus, our results indicate that the nucleoside analog treatment is effectively introducing mutations into the viral genomic LCMV RNA.

Amino acid replacements corresponding to mutations found in control as well as in FU- and AZC-treated LCMV populations are shown in Table 2, which is published as supporting information on the PNAS web site, [www.pnas.org](http://www.pnas.org). All of the amino acid replacements were exclusive to mutagenized virus populations when compared with those observed in the control virus populations. AZC-induced amino acid replacements were more evenly distributed in the three genomic regions, whereas for FU, the L and GP regions accepted fewer amino acid replacements than NP. In particular, at passage 9 of LCMV in 20  $\mu\text{g/ml}$  FU only about 30%

of the mutations found in the L or GP regions led to amino acid replacement compared with the 70% observed for the NP region, indicating that amino acid changes are more tolerated in NP. The fact that only 28% of the mutations found in the L region of passage 9 in 20  $\mu\text{g/ml}$  FU were nonsynonymous indicates that although this region can accept higher number of mutations the majority do not alter the amino acid sequence of the polymerase. Comparison of the degree of acceptability of amino acid substitutions (47) suggests an increase in drastic amino acid substitutions in mutagenized populations.

Our data demonstrate conclusively that an RNA virus can be systematically and efficiently driven into extinction by nucleoside analogs, presumably by introducing mutations in the viral RNA. Although the easiest interpretation of our results is that they indicate that FU drives LCMV quasispecies into extinction because of increased mutagenesis, direct evidence of melting genomic information is still lacking. We postulate that genome mutagenesis will affect primer binding sites used to amplify viral genomes and that mutation frequencies obtained thus reflect viral genomes at the gates of error catastrophe. Because RNA virus populations replicate close to error catastrophe (4–6, 11–14, 39–43, 45, 46), genomes including a substantially larger number of mutations than found in the pre-extinction populations, may have a fleeting existence, and constitute a minority in the hypermutated RNA genome population. Proof of the existence of such molecules on their way to melting of genetic information (4, 11, 13, 46) will require development of analytical techniques to gain access to such replication-incompetent intermediates.

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