CHAPTER

TRENDS IN ANTIVIRAL STRATEGIES

9

CHAPTER CONTENTS

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
APOBEC3G	apolipoprotein B mRNA editing complex 3G
AZA-C	azacytidine
FMDV	foot-and-mouth disease virus
FU	5-fluorouracil
FUTP	5-fluorouridine-triphosphate
GTP	guanosine-5′-triphosphate
HAART	highly active antiretroviral therapy
HCV	hepatitis C virus
HIV-1	human immunodeficiency virus type 1
IFN	interferon
IMPDH	inosine monophosphate dehydrogenase
LCMV	lymphocytic choriomeningitis virus
NGS	next generation sequencing
NLS	nuclear localization signal
PV	poliovirus
Rib	ribavirin
RMP	ribavirin-monophosphate
VSV	vesicular stomatitis virus

9.1 THE CHALLENGE

There is no general procedure that can effectively prevent or control viral infections, at least based on the strategies developed over the last century and a half: vaccination, immunotherapy, chemotherapy, or their combinations. One of the main reasons is that each pathogenic virus to be controlled has some unique features in its interaction with the host cell. Not only for viruses but also for cellular pathogens (bacteria, fungi, and protozoa) it is amply recognized that current practices for prevention and treatment have clear limitations. The difficulties arise also from the evolutionary potential of viral and cellular pathogens that can jeopardize control strategies. For many decades there has been limited awareness of the adaptive potential of pathogens, and preventive and therapeutic designs were implemented ignoring evolution. Different experts have expressed the need to search for new paradigms to approach infectious disease, with the incorporation of Darwinian principles together with concepts from evolutionary ecology, and considering also re-implementation of old methods such as passive antibody therapy (Casadevall, 1996; Stearns, 1999; Williams, 2009; Casadevall and Pirofski, 2015). Only very recently the adaptive potential of pathogens, in particular viral quasispecies dynamics, and its extensions to other pathogenic entities (Chapter 10) have been considered an aspect of the disease control problem.

The challenge to be confronted is symbolically portrayed in Figure 9.1, using one of the ways to depict viral quasispecies throughout this book (lines to depict genomes and symbols on the lines to represent mutations). About 100 million sheets (!) similar to the one shown in the figure are necessary to represent the hepatitis C virus (HCV) genomes found in an acutely infected human liver at a given time point. The precise number, types, and distribution of mutations among genomes varies not only among patients but also as a function of time in each patient. A few minutes later a slightly different image will be produced, with relative proportions of different genomes, and the location of several mutations

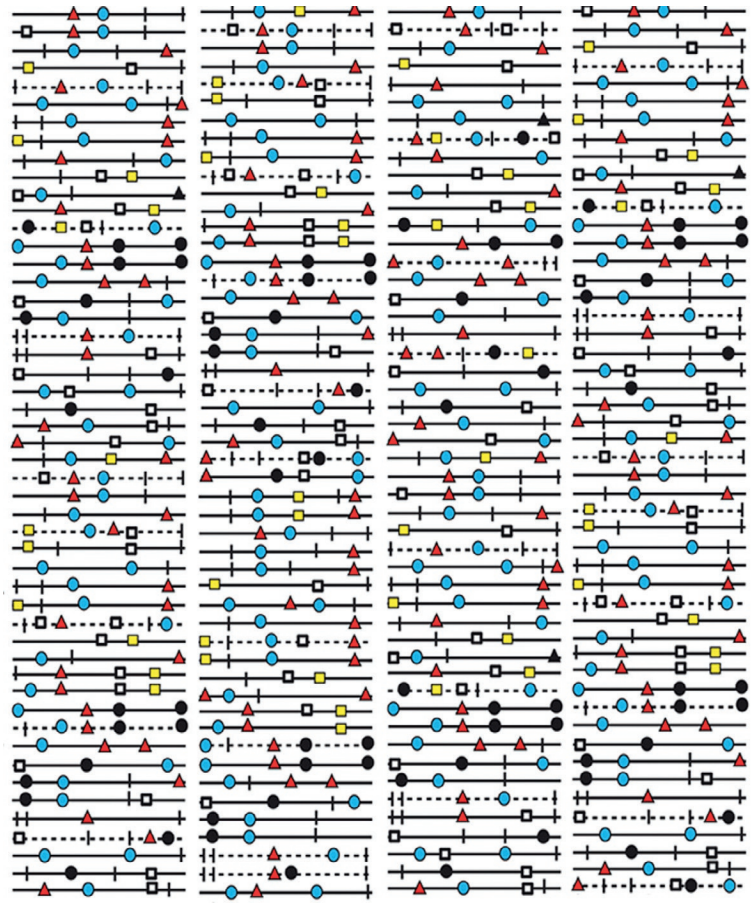

FIGURE 9.1

A scheme of quasispecies intended to stress the challenge of controlling replication of an immense and diverse entity. Each horizontal line represents a viral genome and symbols on the lines symbolize mutations, in a typical depiction of quasispecies used throughout the book. The discontinuous lines represent genomes with five or six mutations considered in this example a sufficient number to severely decrease fitness. Their fate is to attain very low frequency levels or be eliminated from the evolving population. About 100 million of displays similar to the one shown in this page are needed to depict the hepatitis C viral genomes present in an individual acutely infected with the virus.

modified. The dynamic mutant cloud here drawn on the basis of estimated HCV population numbers (and probably with underrepresentation of the number of mutations per genome!) is equally applicable to the majority of RNA viruses and error-prone DNA viruses. It should be quite obvious to the reader of previous chapters that there is a nonzero probability that mutations present in the mutant spectrum may contribute to drug resistance, immune evasion, or fitness enhancement. Even if they are hidden as a tiny minority, they are ready to be selected. The challenge is clear.

9.1.1 VIRUS AS MOVING TARGETS

The potential deleteriousness of mutational load (excessive number of mutations in the same genome) is symbolized in Figure 9.1 by the genomes drawn as discontinuous lines that contain five mutations, here assumed to be a sufficiently large number to decrease fitness and, therefore, the frequency of their potential descendants in subsequent replication rounds. Quasispecies dynamics imposes, however, that those genomes that move from a high frequency to a low frequency level be replaced by newly arising mutants that display higher fitness. The mutant cloud is highly dynamic with newly arising genomes incessantly exposed to the scrutiny of selection and the lottery of random drift. The image here portrayed through mutations is even more complex if we add recombination and genome segment reassortment in the case of viruses with segmented or multipartite genomes (Chapters 2 and 3). From the point of view of antiviral interventions, viruses are true "moving targets," in the sense that the repertoire of variants that we should inhibit at one time point is not exactly the same to be inhibited at a subsequent time point. The difference may be irrelevant regarding the efficacy of an antiviral treatment, or it may not. The impact of the dynamic change is unpredictable. Applied to clinical practice, it means that a specific inhibitor combination may be effective for many infected individuals for a long time, or only for a few individuals for a short time, with a range of possible intermediate outcomes. The new antiviral strategies discussed in this chapter take into consideration the moving target feature of the viruses to be controlled, and they have been proposed by an increasing number of experts aware of the quasispecies challenge.

9.2 PRACTICED AND PROPOSED STRATEGIES TO CONFRONT THE MOVING TARGET CHALLENGE WITH ANTIVIRAL INHIBITORS

The basic statistical considerations that justify the need to use multiepitopic vaccines to protect against variable viruses characterized by quasispecies behavior (keep in mind Figure 9.1) were discussed in Section 8.3 of Chapter 8. In this chapter, we focus on antiviral therapies; immunotherapy is only mentioned as a potential ingredient of combination therapies with antiviral inhibitors.

The systematic selection of drug (or multidrug)-resistant mutants in viral populations has encouraged the design of antiviral strategies intended to avoid viral breakthrough and treatment failure. The main options in clinical practice or under investigation using nonmutagenic and mutagenic antiviral inhibitors are summarized in Box 9.1. None of the listed strategies is totally free of the problem of selection of drug-resistant mutants, but the proposals are intended to avoid or delay their selection. Next, we discuss the major features of each of the six suggestions listed in Box 9.1.

9.2.1 COMBINATION TREATMENTS

Studies on the advantage of the combined administration of two drugs were pioneered by H.J. Eggers and I. Tamm (Eggers and Tamm, 1963; Eggers, 1976). Combination therapy applied to human immunodeficiency virus type 1 (HIV-1) has been the great success for the control of acquired immunodeficiency syndrome (AIDS) that has drastically reduced AIDS-related mortality. This type of treatment for HIV-1 is termed highly active antiretroviral therapy (HAART) and it is ideally implemented with three different antiretroviral agents. HAART efficacy has steadily improved due to availability of new inhibitors directed at different HIV-1 targets. Success is only partial due to side effects (that may be

BOX 9.1 SOME ANTIVIRAL STRATEGIES TO CONTROL VIRAL QUASISPECIES

Based on Nonmutagenic Inhibitors

- Combination treatments. Use of two or more inhibitors directed to independent viral targets.
- Splitting the treatment into an induction and a different maintenance regimen.
- Targeting cellular functions.
- Use of drugs that stimulate the host innate immune system.
- Combined use of immunotherapy and chemotherapy.

Based on Mutagenic Agents

- Lethal mutagenesis in their two modes of sequential and combined administration of inhibitors and mutagens.

derived from off-target activities of the drugs), incomplete patient adherence to the treatment, selection of multidrug-resistant viral variants and, above all, to the retroviral nature of HIV-1 that renders virus extinction from the organism a difficult endeavor. In retroviruses and hepadnaviruses, the viral DNA is not only hidden from antiviral drugs but it can serve as an archive of genomes that can reactivate replication upon treatment discontinuation. If combination therapy could be coupled with an efficient elimination of the proviral reservoirs in host DNA (De Crignis and Mahmoudi, 2014), HIV-1 could probably be eliminated from the infected organism, as achieved in the case of HCV infections in some patients.

The general advantage of combination therapy over monotherapy is a consequence of quasispecies dynamics, and it has been amply evidenced by clinical practice, and supported by straightforward statistical considerations and theoretical models of virus dynamics (Domingo, 1989; Domingo and Holland, 1992; Ho, 1995; Bonhoeffer et al., 1997; Pol et al., 1999; Ribeiro and Bonhoeffer, 2000; Le Moing et al., 2002; Van Vaerenbergh et al., 2002; Domingo et al., 2008; Müller and Bonhoeffer, 2008; Nijhuis et al., 2009) (see Section 8.4 in Chapter 8). Furthermore, use of combination therapies conforms to the "hit early, hit hard" dictum of D.D. Ho, justified in Section 8.10 of Chapter 8. There is the misconception (among some experts, but mainly among politicians!) that only patients in advanced phases of a viral infection (i.e., only precirrhotic or cirrhotic patients infected with HCV) should be treated aggressively with drug combinations. This is not what evolutionary virology teaches us: patients should be treated as strongly as possible and as early as possible, provided side effects can be controlled. Viruses should be given no chance to walk in sequence space in search of adaptive pathways (Chapter 3). Adequate combination treatments can fulfill such purpose.

9.2.2 SPLIT TREATMENTS

A second proposed strategy is to divide an antiviral treatment in two steps: an induction and a maintenance step (von Kleist et al., 2011). It is based on a theoretical model developed for the treatment of HIV-1 infections. The key argument is that when a treatment has to be changed after confirmation of treatment failure (virus rebound), mutants resistant to the ineffective drug have had the opportunity to replicate in the patient, increasing the viral load and supplying a proviral archive with inhibitor-resistant latent viruses, thus excluding the drug as a component of future combinations. A treatment with an induction regimen should be followed by a shift to a maintenance regimen at a point in time

in which the second drug finds a low viral load and limited numbers of mutants resistant to the initial treatment. Critical issues for the implementation of this proposal are the timing of treatment switch, the decrease of viral load as a result of the induction regimen, and the genetic and phenotypic barriers to the maintenance regimen. Clinical trials are needed to explore this interesting proposal.

9.2.3 TARGETING CELLULAR FUNCTIONS

Many cellular functions are needed to complete any step of a virus replication cycle. A good deal of research in virology has as its main objective to identify and characterize cellular functions that participate in virus entry into the cell, intracellular multiplication, or release from cells. Since cellular proteins cannot vary in response to the presence of an inhibitor (at least within the time frame of a viral infection), an obvious thought is to administer inhibitors of those cellular functions (often proteins) that are needed to sustain viral replication; such inhibitors should suppress viral replication without selection of inhibitor-resistant mutants (Geller et al., 2007; Hopkins et al., 2010; Garbelli et al., 2011; Kumar et al., 2011; Vidalain et al., 2015). Despite potential benefits, two major problems may be encountered with antiviral agents directed to cellular proteins: (i) toxic effects derived from suppression or alteration of activities in which the target protein is involved and (ii) selection of viral mutants that are insensitive to the presence of the inhibitor, despite the inhibitor not being directed to the virus. Insensitivity may come about by at least three different mechanisms. If the cellular protein which is the target of the inhibitor forms a complex with a viral protein in the course of viral replication, amino acid substitutions in the viral protein may permit progression of the infection in the presence of the inhibitor. This is the case of HCV resistance to the nonimmunosuppressive cyclophilin inhibitor SCY-635. NS5A interacts with cyclophilins and with NS5B; amino acid substitutions in NS5A (T17A, E295K, and V44A) and in NS5B (T77K and I432V) decrease the sensitivity of the virus to SCY-635, although the precise molecular mechanism of resistance has been debated (Chatterji et al., 2010; Sarrazin and Zeuzem, 2010; Delang et al., 2011; Kwong et al., 2011; Vermehren and Sarrazin, 2011).

When inhibitors are targeted to the cellular receptor for a virus, mutants may be selected that can enter cells through an alternative receptor. This has been documented with HIV-1 that can use coreceptor CCR5, CXCR4, or both (dual-tropic viruses). A modified RANTES [*r*egulated on *a*ctivation, *n*ormal *T* cell *e*xpressed, and *s*ecreted; also termed chemokine (C–C motif) ligand 5 or CCL5] selected coreceptor switch variants in a SCID (*s*evere *c*ombined *i*mmuno*d*eficiency) mouse model (Mosier et al., 1999). The mutants with amino acid substitutions in loop V3 of Env protein were selected to use CXCR4 rather than CCR5 as coreceptor (see also Section 4.4 in Chapter 4).

An alternative mechanism to overcome the inhibition of a cellular protein is that viral mutants are selected that can utilize the cellular protein in complex with the inhibitor. This is the case of HIV-1 mutants resistant to small coreceptor CCR5-binding inhibitors; mutants with amino acid substitution in the V3 loop region or elsewhere in Env can use either free or inhibitor-bound CCR5 to enter cells, with an efficiency that depends on the CCR5 expression level and the host cell type (Pugach et al., 2007, 2009). These examples illustrate the multiple pathways that viruses can exploit to overcome inhibitors directed to cellular proteins. They constitute additional evidence that "abundant sources of genetic variation exist for viruses to learn new tricks, …" emphasized by J. Lederberg in connection with viral disease emergence and reemergence (quoted also in Section 7.7.1 of Chapter 7). Since many viruses can use alternative receptors for entry into cells (Section 4.4 in Chapter 4), inhibitors directed to viral receptors may promote selection of virus subpopulations that can use a different receptor.

9.2.4 USE OF DRUGS THAT STIMULATE THE HOST INNATE IMMUNE SYSTEM

Some inhibitors of enzymes of the *de novo* pyrimidine biosynthesis pathway (DD264, bequinar, and A3) stimulate the innate immune response, and behave as broad-spectrum antiviral inhibitors (Lucas-Hourani et al., 2013; Munier-Lehmann et al., 2015; Vidalain et al., 2015). The observed effect is one among other connections that have been established between nucleotide and DNA metabolism and immune stimulation (Motani et al., 2015). For some of the pyrimidine biosynthesis inhibitors, additional mechanisms of antiviral activity might be involved, as in the inhibition of lymphocytic choriomeningitis virus (LCMV) replication and transcription by A3 (Ortiz-Riano et al., 2014). Stimulation of the innate immune response may restrict the selection of escape mutants because the virus must mutate at several sites to overcome the different branches of the response, as is the case with interferon (IFN) resistance (Perales et al., 2014). Multifactorial antiviral responses increase the genetic and phenotypic barriers to resistance (Section 8.4.2 in Chapter 8).

9.2.5 COMBINED USE OF IMMUNOTHERAPY AND CHEMOTHERAPY

An extension of the advantage of combination therapy to decrease the selection of antiviral-resistant mutants consists in the combined use of immunotherapy (administration of neutralizing antibodies or other means of immune stimulation such as vaccination) together with antiviral inhibitors. The concept was pioneered by R.G. Webster and colleagues, and was proposed as a strategy for the control of influenza viruses (Webster et al., 1985). The authors showed that the simultaneous administration of inactivated H5N2 vaccine and the inhibitor amantadine conferred protection against H5N2 influenza virus A/Chick/Pennsylvania/83 in chickens (Webster et al., 1986). Related notions have been investigated with other viruses and different components of the immune response (Seiler et al., 2000; Li et al., 2005). However, additional model *in vivo* experiments with animals and clinical trials with patients are necessary to investigate the effectiveness of combined immunotherapeutic approaches.

As a general outlook on the strategies summarized in previous sections, the potential of combining two (or even more) of the proposals listed in Box 9.1 is encouraging, provided off-target effects of drugs or immune interventions can be controlled and side effects minimized. A trend toward "complex" treatment protocols is the expected response to the adaptive capacity of viral quasispecies. The introduction of mutagenic agents in antiviral designs is an important departure that exploits one of the corollaries of quasispecies behavior: the error threshold relationship (introduced in Section 3.6.3 of Chapter 3). It is the basis of lethal mutagenesis discussed next.

9.3 LETHAL MUTAGENESIS AND THE ERROR THRESHOLD

Lethal mutagenesis is defined as the process of viral extinction due to an excess of mutations in the viral genome. J.J. Holland and colleagues pioneered studies on the adverse effects of several chemical mutagens on the yield of infectious poliovirus (PV) and vesicular stomatitis virus (VSV) in cell culture (Holland et al., 1990). The term lethal mutagenesis was first proposed by L. Loeb, J.I. Mullins, and colleagues in a study of the loss of replicative potential of HIV-1 upon multiplication in human CEM cells in the presence of the deoxynucleoside analog 5-hydroxydeoxycytidine (5-OH-dC) (Loeb et al., 1999). The experimental design of lethal mutagenesis was inspired in the error threshold relationship derived from the basic equation of quasispecies dynamics (Eigen and Schuster, 1979; Swetina and Schuster,

1982; Nowak and Schuster, 1989; Schuster, 2016). Initially elaborated on simple (single peak) fitness landscapes, quasispecies theory has as one of its main corollaries that the stability of genetic information during a replicative process is dependent on two parameters: the error rate during replication and the amount of genetic information to be maintained. The basic equations that describe the relationship between the maximum tolerable mutation rate (μ_{max}) to ensure the transfer of genetic information to next generations of genomes with length ν, are included in Figure 9.2. They are the most relevant to an application of the error threshold concept to virology (Eigen, 2002; Schuster, 2016). In the equations, an important variable is the superiority of the master sequence over its surrounding mutant spectrum, denoted by σ_m. Consistent with σ_m being in the numerator of the equations, virologists will intuitively understand that a high superiority of the dominant sequence means a strong settlement of the virus with a well-adapted master sequence and its surrounding cloud in that environment. In consequence, a higher mutational input is necessary to destabilize the distribution.

There are additional factors that can modify the stability of a mutant distribution and the position of the error threshold; the mathematical justification and practical implications for virology have been detailed (Schuster, 2016). Of particular relevance to virus population stability is the influence of the fitness landscape in maintenance of genetic information. The first noteworthy result of the theoretical studies is that an error threshold is present in realistic rugged fitness landscapes, as those proposed to best describe viruses on the basis of experimental evidence (Section 5.3 in Chapter 5). The mathematical derivation of this important conclusion has been obtained by Schuster (2016). Second, the position of the error threshold moves toward lower mutation rates when the ruggedness of the fitness landscape (represented by a parameter that consists of a band of fitness values) is increased. How replication of viruses in variable fitness landscapes (e.g., under variable environmental conditions) versus a constant environment may affect the ease of extinction by increased mutagenesis is a largely unexplored question.

9.3.1 RECONCILIATION OF THEORY AND EXPERIMENT: A PROPOSAL

Several theoretical models have been presented to explain lethal mutagenesis of viruses (review in Tejero et al., 2016). The models are conceptually diverse, and at times with remarkably counterintuitive proposals. Some deny a connection between the error threshold of quasispecies theory and the extinction of viruses by enhanced mutagenesis. In one of the models discussed by H. Tejero and colleagues, it was suggested that error catastrophe could not occur in the presence of lethal genotypes, a proposal that was considered peculiar by experimentalists, and that it was proven incorrect by Takeuchi and Hogeweg (2007) [see also references in that publication and in Tejero et al. (2016)]. Other models that have suggested that lethal mutagenesis is unrelated to the error threshold have defined an extinction threshold to mean the mutation rate at which a viral population goes extinct. A rather counterintuitive proposal is that the error threshold is caused by the "survival of the flattest," which means dominance of genomes with low replicative capacity and high tolerance to mutations (robustness) that would hinder virus extinction (Tejero et al., 2011) (see Section 5.7 in Chapter 5 on the advantage of the flattest in a fitness landscape).

C. Perales and I have carefully reviewed the main experimental results obtained in our and other laboratories on the molecular events that accompany the transition toward virus extinction, and have tried to harmonize the experimental observations with the most realistic and significant theoretical models reviewed by Tejero et al. (2016). The main stream of experimental results can be summarized as follows. The first studies, that were an extension of those carried out in J.J. Holland's laboratory,

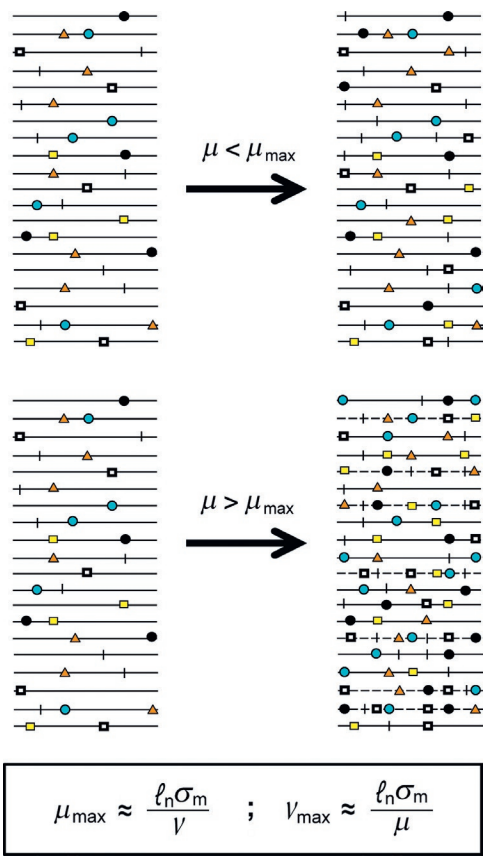

FIGURE 9.2

A representation of virus entry into error catastrophe and the error threshold relationships of quasispecies theory. The mutant spectrum at the top replicates with a mutation rate (μ) below the maximum compatible with maintenance of genetic information (μ_{max}), and the population remains stable. The mutant spectrum below the first one replicates with a mutation rate (μ) that exceeds the highest tolerable (μ_{max}) for maintenance of the genetic information. As a consequence, there is a progressive deterioration of viral functions and genome replication, represented here by genomes drawn as discontinuous lines. Their number increases as replication proceeds under conditions of $\mu > \mu_{max}$, until the system collapses into total loss of information (loss of virus identity and transition into replication-incompetent sequences). The box below the mutant distributions includes the basic mathematical formulae of the error threshold, in which σ_m is the selectivity or superiority of the master sequence over its mutant spectrum, ν is the chain length of the replicating genome, and ν_{max} is the maximum length whose information can be maintained when replicating with mutation rate μ. For the mathematical derivation of the error threshold relationships, see Schuster (2016). See text for the connection between the error threshold and lethal mutagenesis.

confirmed a connection between a mutagenic activity and decrease of viral infectivity and showed that low viral load and low viral fitness favored virus extinction (Loeb et al., 1999; Sierra et al., 2000). Then, following the important discovery that the nucleoside analog ribavirin (1-β-D-ribofuranosyl-1-*H*-1,2,4-triazole-3-carboxamide) is mutagenic for PV (Crotty et al., 2000) it was observed that ribavirin decreased PV-specific infectivity (the ratio of infectivity to the amount of viral RNA) (Crotty et al., 2001). A decrease of specific infectivity, together with increase of mutation frequency is now considered the standard way to distinguish lethal mutagenesis from mere inhibition (Box 9.2). [This is not to mean that decreases of specific infectivity are exclusive of lethal mutagenesis. They have been also documented as a result of codon deoptimization (Section 4.3 of Chapter 4) and in viral clones subjected to many plaque-to-plaque transfers (Section 6.5.2 in Chapter 6)].

In a subsequent study, A. Grande-Pérez and colleagues documented that treatment of cells persistently infected with LCMV with 5-fluorouracil [5-Fluoro-1*H*,3*H*-pyrimidine-2,4-dione (FU)] resulted in a decay of infectivity that preceded the decay of viral RNA (see Section 9.4 for the mutagenic mechanisms of ribavirin and FU). This result suggested that a class of replication-competent RNA that did not lead to infectious virus was generated during the FU-mediated transition toward viral extinction. A theoretical model developed by S. Manrubia predicted that such defective genomes that were termed defectors were required to achieve LCMV extinction with a limited mutagenic intensity by FU (Grande-Pérez et al., 2005b). The proposal that loss of viral infectivity is due to the action of defective genomes produced by the mutagenic agent is termed the lethal defection model of virus extinction, and it is consistent with several observations on extinction of other viruses (reviewed in Domingo et al., 2012). A diagnostic test is that loss of infectivity precedes loss of viral RNA (Figure 9.3). Lethal defection can be regarded as an extreme outcome of interfering interactions that are exerted among components of the mutant spectrum when their mutational load increases (compare with Section 3.8 of Chapter 3).

The studies by A. Grande-Pérez, S. Manrubia, and colleagues distinguished two pathways that viruses can follow when subjected to mutagenesis: lethal defection at low mutagenic intensities, and overt lethality at high mutagenic intensities. Figure 9.4 recapitulates our understanding of the steps involved in mutagenesis-driven virus extinction, with the important qualification that no sharp boundary exists

BOX 9.2 MAIN OBSERVATIONS ON VIRAL EXTINCTION BY MUTAGENIC AGENTS

- Low viral load and low viral fitness favor extinction.
- During the transition toward extinction, the viral population:
  - Decreases its specific infectivity.
  - Increases its mutant spectrum complexity (movement toward usually unfavored regions of sequence space).
  - Maintains an invariant consensus sequence.
- Reduction in viral load *per se* is not the mechanism of virus extinction. A load decrease by a mutagenic agent may extinguish the virus while the same decrease with an inhibitor may not.
- Viruses displaying different replicative features respond to mutagenic agents in a very similar way: the strategy is of general applicability provided a virus-specific mutagenic agent is available.
- The use of mutagens as antiviral agents has been validated *in vivo*.

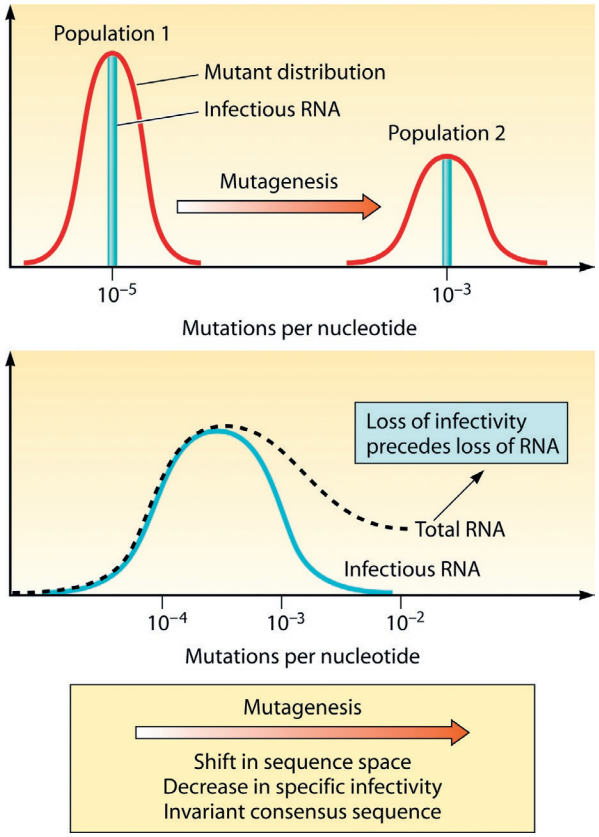

FIGURE 9.3

A summary of the main experimental observations upon mutagenesis of a viral population. Top: mutagenesis results in an expansion of mutant spectrum (red Gaussian distribution, that may be skewed in real populations), a 100-fold increase in the average number of mutations per nucleotide, and a decrease of infectious RNA (blue thin column) (specific values depend on each experimental system). Bottom: the increase in the average number of nucleotides leads to maintenance of viral RNA (discontinuous curve) despite loss of infectivity (blue curve). The box at the bottom underlines the major changes underwent by the viral genomes as the result of mutagenesis; these points are also included in Box 9.2.

Figure reproduced from Domingo et al. (2012), with permission from the American Society for Microbiology, Washington DC, USA.

between the lethal defection and the overt lethality phases. Studies with foot-and-mouth disease virus (FMDV) have provided further support to the lethal defection phase of extinction. Defective FMDV RNAs inhibited replication of standard FMDV RNA in a specific manner when co-electroporated into cells (Perales et al., 2007). Specificity means that the defective FMDV RNAs did not inhibit replication of the related encephalomyocarditis viral RNA. Specificity was also evidenced by the loss of interfering activity of a defector genome by the introduction of a mutation that prevented RNA replication. The requirement of replication of the defective RNAs suggests that a sufficient amount of expressed

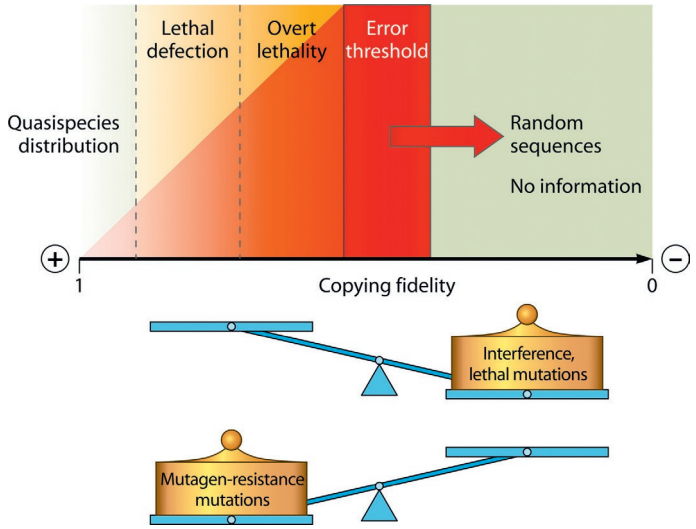

FIGURE 9.4

Scheme of the steps involved in virus extinction by enhanced mutagenesis. The graph includes a basic element of quasispecies fidelity: the horizontal black arrow that spans the entire possible range of copying fidelity values: from value 1 (perfect copying fidelity, no mutations; incompatible with evolution of life) to 0 (complete infidelity, mutation as the norm; incompatible with maintenance of genetic information). On top of the horizontal axis, four successive recognized steps, that are reached with decreasing copying fidelity, are represented: the region of maintenance of a dynamic quasispecies, the region of lethal defection, the region of overt lethality and, finally, the crossing of the error threshold (red arrow). The very last transition can be visualized as the viral genomic sequences having degenerated into random sequences; that is, a transition from information to no-information. The balances drawn at the bottom symbolize influences that may either favor or prevent virus extinction.

Figure reproduced from Domingo et al. (2012), with permission from the American Society for Microbiology, Washington DC, USA.

proteins is needed for interference (Perales et al., 2007). These results are consistent with enhanced and also specific interfering activity exerted by mutagenized FMDV RNA (González-López et al., 2004). Thus, the available evidence suggests that mutant RNAs—whose frequency and mutational load increase as the mutagenesis proceeds—adversely affect the replication of nonmutated RNAs that coexist in the same replicative ensemble. This is the major molecular event that has been recognized in the lethal defection phase of Figure 9.4.

The infectious FMDV RNA that can be retrieved from a mutagenized population by dilution and plaque isolation display decreases in replicative fitness (up to 200-fold lower infectivity) relative to the parental, nonmutagenized virus (Arias et al., 2013) associated with eightfold increase in mutation frequency. This result reinforces the complexity of events during lethal mutagenesis, confirms that fitness may be impaired in clones that survive transiently the mutagenic activity, and suggests an overlap between the lethal defection and overt lethality phases drawn in Figure 9.4.

We can now consider theoretical models in the light of the experimental results. First, the proposed interference by substituted, *trans*-acting proteins is in line with the early descriptions of fitness

deterioration of cells due to the collapse of interdependent nucleic acid and protein *trans* networks in connection with the process of aging (Orgel, 1963). In the case of lethal defection, the effects of mutations have to be calibrated keeping in mind the multifunctional nature of viral proteins (Section 3.8.1 in Chapter 3). When a protein is defective it can jeopardize the activities of any other proteins that interact with it: a *trans* network can collapse by a domino effect. The possible influence of the topology of the network of interactions among genomes for maintenance of population stability is a largely unexplored possibility which is briefly addressed in the closing Chapter 10.

The notion that viral mutagenesis promotes drift in sequence space was shown by a direct amplification of A, U-rich genomic sequences of FMDV subjected to ribavirin mutagenesis (Perales et al., 2011b). The main effect of ribavirin was to accelerate the occupation of A, U-rich regions of sequence space, presumably due to the tendency of this purine analog to produce an excess of G → A and C → U transitions (Section 9.4.1). Analysis of the numbers and types of mutations suggests that the A, U-enriched portion of sequence space is detrimental to viral fitness. Movements toward unfavorable regions of sequence space are also suggested by mutant spectrum analyses of FMDV subjected to FU mutagenesis and other viruses subjected to other mutagenic agents (Grande-Pérez et al., 2002, 2005a; Agudo et al., 2008; Ortega-Prieto et al., 2013).

In view of the above evidence, any theoretical model of lethal mutagenesis that proposes a delocalization of the genome population in sequence space fits the experimental results of extinction. Specifically, models based on the advantage of the flattest that predict absence of extinction (Tejero et al., 2016) in reality predict the extinction of a real virus. This is because the mutagenesis-driven, astray walk in sequence space in the absence of a dominant master sequence should produce increased number of defective genomes (lethal defection) in unfavorable regions of sequence space (such as the A, U-rich regions promoted by ribavirin). The net result should be not only lethal defection but also an increasingly frequent hitting of lethal portions of the space (overt lethality phase in Figure 9.4). Thus, any theoretical models based on genome sequence delocalization fit with the experimental observations (Perales and Domingo, 2016). How such delocalization can be turned into an antiviral strategy is discussed in the next sections.

9.4 VIRUS EXTINCTION BY MUTAGENIC AGENTS

The pioneer experiments by J.J. Holland and colleagues demonstrated the adverse effects of mutagenic agents—including the base analog FU and the nucleoside analog 5-azacytidine [4-amino-1-β-D-ribofuranosyl-1,3,5-triazin-2(1*H*)-one (5-AZA-C)]—on the production of infectious PV and VSV progeny (Holland et al., 1990; Lee et al., 1997). These investigations were followed by several others that examined the effect of base and nucleoside analogs on virus survival. A few studies with animal viruses are summarized in Table 9.1 to illustrate that viruses displaying diverse replicative strategies (positive and negative strand RNA viruses and retroviruses) are vulnerable to increases in mutation rate. The investigations in cell culture or animal hosts employ mutagenic base or nucleoside analogs which are converted intracellularly into the nucleoside-triphosphate forms. The latter can be incorporated into RNA that in the next round of template copying will give rise to misincorporations (point mutations), and in some cases to RNA chain termination. Mutagenesis is mainly due to ambiguous pairing between the mutagenic base and standard nucleotides, with the formation of Watson-Crick or wobble base pairs. Some base pair structures of FU with A or G are drawn in Figure 9.5 (Sowers et al., 1988;

Table 9.1 Some Studies on Lethal Mutagenesis of Viruses

Virus[a]	Base or Nucleoside Analog[b]	Main Observations	Reference
FMDV	FU 5-AZA-C	Extinction favored by low viral load and low viral fitness	Sierra et al. (2000), Pariente et al. (2001)
FMDV	Rib	Enhanced mutagenesis eliminated virus from persistently infected cells	Airaksinen et al. (2003)
PV	Rib	Evidence of Rib-mediated error catastrophe of PV	Crotty et al. (2001)
HCV	Rib	Rib increased the mutation frequency in conserved regions of a HCV replicon genome	Contreras et al. (2002)
GBvB	Rib	Evidence of error-prone replication induced by Rib in cell culture, but no significant reduction of viremia *in vivo* (tamarin model). RTP incorporated in viral RNA can induce misincorporations and an elongation block	Lanford et al. (2001), Maag et al. (2001)
WNV	Rib	Error-prone replication and transition to error catastrophe were dependent on the host cell line	Day et al. (2005)
Hantaan virus	Rib	Rib-induced high mutation frequency in S segment. A range of Rib concentrations revealed a nonlinear fit of mutation frequency and mutagen concentration	Severson et al. (2003), Chung et al. (2007)
LCMV	FU	Largest increases in mutation frequency did not predict virus extinction. Virus extinction occurred without modification of the consensus sequence	Grande-Pérez et al. (2002, 2005a)
LCMV	Rib	Mutagenic activity documented in cell culture	Moreno et al. (2011)
IV	T-705	First evidence that the broad-spectrum antiviral agent T-705 (favipiravir) can act as a lethal mutagen	Baranovich et al. (2013)
IV	FU 5-AZA-C Rib	Effective lethal mutagenesis of H3N2 and H1N1 IVs. Evidence of high barrier to resistance	Pauly and Lauring (2015)
HIV-1	5-AZA-C	Lethal mutagenesis during reverse transcription is the main antiviral effect of 5-AZA-C, following reduction to the deoxycytidine form	Dapp et al. (2009)

[a] The virus abbreviations are FMDV, foot-and-mouth disease virus; GBvB, GB virus B; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; LCMV, lymphocytic choriomeningitis virus; PV, poliovirus; WNV, West Nile virus.
[b] The abbreviations for drug names are 5-AZA-C, 5-azacytidine; FU, 5-fluorouracil; Rib, ribavirin; T-705, favipiravir.

Yu et al., 1993), and of ribavirin and T-705 (favipiravir) (5-Fluoro-2-oxo-1*H*-pyrazine-3-carboxamide) in Figure 9.6 (Crotty et al., 2000; Jin et al., 2013) (see Section 2.2 in Chapter 2 for the standard Watson-Crick and some wobble base pairs).

The possible point mutations generated as a consequence of FU or ribavirin incorporation during viral genome replication are depicted in Figures 9.7 and 9.8, respectively. The figures represent the

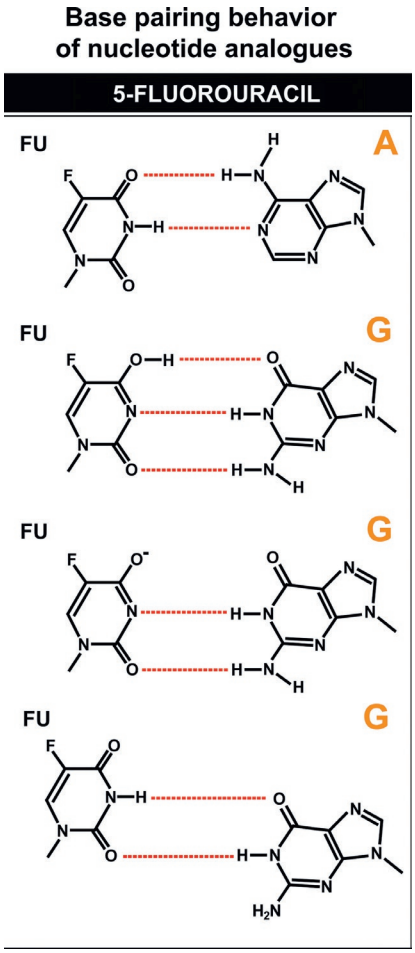

FIGURE 9.5

Some of the possible base pairs between 5-fluorouracil (FU) and the standard nucleotides A and G. Hydrogen bonds are indicated by discontinuous red lines; sugar and phosphate residues are not included.

most frequent case of the successive analog incorporation and subsequent events upon copying of positive and negative strand viral RNA, although the pathways are equally applicable to DNA in the case of deoxynucleotide analogs. The schemes can serve as a guide to anticipate the possible mutation types induced by other analogs provided their base pairing behavior has been investigated by physicochemical procedures, considering that mutation preferences may be modified by environmental factors.

The mutant repertoire produced by any nucleotide analog may be influenced by the structure of the viral polymerase [similar in general shape, but differing in critical molecular details (see Section 2.6 of Chapter 2 on polymerase structure and fidelity mutants, and Section 9.6 on subtle interactions in viral polymerases to confer resistance to mutagenic nucleotide analogs)]. For several viruses, the mutant

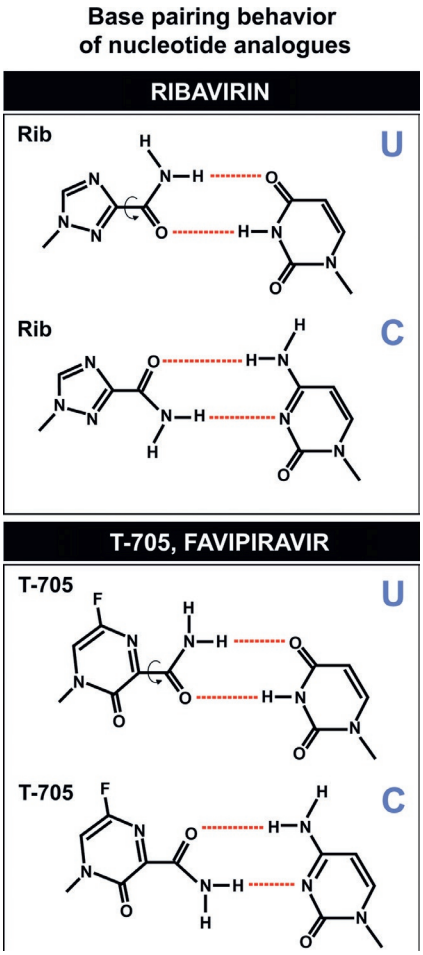

FIGURE 9.6

Some of the possible base pairs between ribavirin (Rib) or favipiravir (T-705) and the standard nucleotides U and C. Hydrogen bonds are indicated by discontinuous red lines; sugar and phosphate residues are not included. The rotation about the carboxamide bond that contributes to alternative pairing with U or C is indicated by the small circular arrow.

spectra produced upon replication in the presence of FU include an excess of A → G and U → C over G → A and C → U transitions (Sierra et al., 2000; Grande-Pérez et al., 2002; Ruiz-Jarabo et al., 2003; review on FU antiviral activity and mutagenesis in Agudo et al., 2009). The bias in favor of A → G and U → C mutations suggests that FU behaved as U rather than C when incorporated by the viral polymerases and that once FU was a template residue it tended to behave as C as rather than U (Figure 9.7). Studies with several viruses have indicated that ribavirin mutagenesis yields mutant spectra with an excess of G → A and C → U over A → G and U → C (Figure 9.8) (Crotty et al., 2001; Airaksinen et al.,

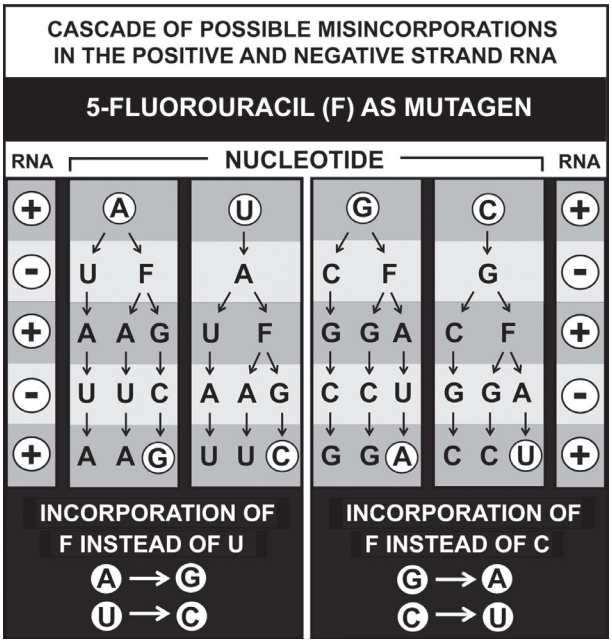

FIGURE 9.7

Mutations produced by 5-fluorouracil (F) as a result of its incorporation into viral RNA. The RNA polarity is indicated on the left (+, positive strand or genomic RNA in the case of positive strand RNA viruses; –, negative strand or complementary RNA; in the case of negative strand RNA viruses the genomic RNA is of negative polarity). Downward arrows indicate standard base copying and F incorporation. The block on the left explains the consequences of incorporation of F instead of U either in the minus strand (second row) or in the plus strand (third row). The block on the right indicates the consequences of incorporation of F instead of C either into the minus strand (second row) or into the plus strand (third row). The boxes at the bottom indicate the types of mutations expected in the mutant spectrum (see text for references).

2003; Chung et al., 2007; Agudo et al., 2010; Moreno et al., 2011; Dietz et al., 2013; Ortega-Prieto et al., 2013; among other studies; as reviews of the mutagenic activity of ribavirin see Crotty et al., 2002; Beaucourt and Vignuzzi, 2014).

The variation of mutational spectrum evoked by ribavirin depending on the polymerase and environmental factors is illustrated by a study of C.B. Jonsson and colleagues with Hantaan virus. They observed a much higher frequency of G→A than C→U mutations induced by ribavirin in this virus (Chung et al., 2007), as detected also with West Nile virus (Day et al., 2005). The effect of a mutagenic nucleotide analog may be influenced by the position it occupies in the template. This was shown in the early site-directed mutagenesis experiments of bacteriophage Qβ RNA performed by R. Flavell, C. Weissmann, and colleagues (Chapter 3). When the pyrimidine analog N^4-hydroxy CMP was present at the extracistronic position 15, it directed the incorporation of GMP slightly more efficiently than AMP, while at position 39 incorporation of AMP was threefold higher than GMP (compare Flavell et al., 1974 and Domingo et al., 1976).

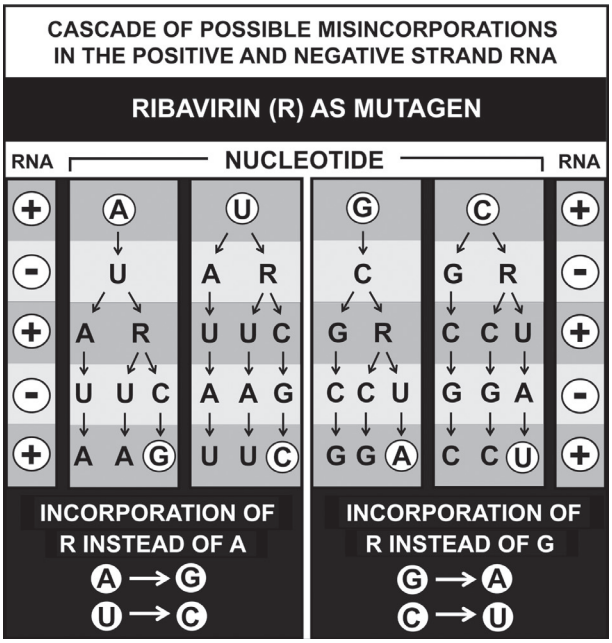

FIGURE 9.8

Mutations produced by ribavirin (R) as a result of its incorporation into viral RNA. The RNA polarity is indicated on the left (+, positive strand or genomic RNA in the case of positive strand RNA viruses; –, negative strand or complementary RNA; in the case of negative strand RNA viruses the genomic RNA is of negative polarity). Downward arrows indicate standard base copying and R incorporation. The block on the left explains the consequences of incorporation of R instead of A either into the minus strand (second row) or into the plus strand (third row). The block on the right indicates the consequences of incorporation of R instead of G either into the minus strand (second row) or into the plus strand (third row). The boxes at the bottom indicate the types of mutations expected in the mutant spectrum (see text for references).

9.4.1 THE SEARCH FOR NEW MUTAGENIC NUCLEOTIDE ANALOGS

New nucleotide analogs are currently being investigated as potential lethal mutagens for viruses (Harki et al., 2002, 2006, 2007; Graci and Cameron, 2004; Beach et al., 2014; Dapp et al., 2014; Vivet-Boudou et al., 2015; among other studies). There is active research to apply drugs (or its derivatives) used in antibacterial or anticancer therapy to lethal mutagenesis of viruses, in a strategy known as drug repositioning or drug repurposing, quite extended in current pharmacology. L.M. Mansky and his associates have pioneered such efforts for the search of new antiretroviral agents, as well as the study of new combination therapies based on lethal mutagenesis (Dapp et al., 2012, 2013; Bonnac et al., 2013; Rawson and Mansky, 2014). M.J. Dapp, L.M. Mansky, and colleagues studied the joint effect of 5-AZA-C and apolipoprotein B mRNA editing complex 3G (APOBEC3G) on the mutational spectrum of HIV-1. The results revealed unexpected changes in the mutational trend, particularly an increase in G → A transitions and a decrease of G → C transversions observed with 5-AZA-C alone (Dapp et al., 2009).

The complexities of the interaction among mutagens and between inhibitors and mutagens in infected cells are still poorly understood. A few studies have provided evidence that some sequential inhibitor-mutagen treatments may have an advantage over the corresponding combinations, fundamentally because they avoid the simultaneous presence of a mutagen and inhibitor during viral replication (Section 9.8).

9.5 LETHAL MUTAGENESIS *IN VIVO*: COMPLICATIONS DERIVED FROM MULTIPLE MECHANISMS OF DRUG ACTION—THE CASE OF RIBAVIRIN

Most of the investigations summarized in the previous section were carried out in cell culture. Some animal experiments and a clinical trial have provided the proof of principle of the feasibility of antiviral interventions based on lethal mutagenesis *in vivo*. J.C. de la Torre and colleagues documented that administration of FU to mice prevented the establishment of a persistent LCMV infection in the animals (Ruiz-Jarabo et al., 2003). J.I. Mullins and colleagues carried out the first phase II clinical trial using lethal mutagenesis by administering KP1461 (N4-heptyloxycarbonyl-5,6-dihydro-5-aza-2′-deoxycitidine), the prodrug of KP1212 (5,6- dihydro-5-aza-2′-deoxycitidine), to HIV-1 infected volunteers previously treated with antiretroviral agents (Mullins et al., 2011). No reduction in viral load or increase in average mutation frequencies was noted in the treated patients. However, mutations that likely occurred in HIV-1 in the course of the treatment were predominantly A → G and G → A transitions, as expected from the base pairing behavior of the pyrimidine analog. The study validated lethal mutagenesis as an antiviral approach for human disease.

The purine analog T-705 (favipiravir) (base pairing behavior shown in Figure 9.6) acted as an effective antinorovirus agent in a mouse model, with features diagnostic of lethal mutagenesis: increases of mutation frequency and decreases of specific infectivity (Arias et al., 2014) (Box 9.2). T-705 is an interesting compound because it shares with ribavirin a broad antiviral spectrum of activity. It has proven effective against several viruses *in vivo*, including West Nile infection of mice and hamsters (Morrey et al., 2008), lethal inhalation of Rift Valley fever virus in rats (Caroline et al., 2014), and highly pathogenic H5N1 IV in mice (Kiso et al., 2010). It is still an open question if T-705 acts as a lethal mutagen in these *in vivo* systems as it does with IV in cell culture (Baranovich et al., 2013) and norovirus in cell culture and *in vivo* (Arias et al., 2014).

An interesting possibility is that the broad-spectrum activity of ribavirin and T-705 is associated with the mutagenic properties of these analogs since the main requirement is that their nucleoside-triphosphate forms be incorporated into replicating RNA. Viral polymerases from different viruses share some features of nucleotide recognition and polymerization so that several of them may be vulnerable to the same nucleotide analogs.

Ribavirin has been used for many years in combination with pegylated IFN-α as the standard of care treatment for HCV infections (McHutchison et al., 1998; Cummings et al., 2001; Di Bisceglie et al., 2001). It is not clear whether lethal mutagenesis is part of the anti-HCV activity of ribavirin, with some studies favoring a mutagenic activity on the virus and others not (Gerotto et al., 1999; Querenghi et al., 2001; Sookoian et al., 2001; Dixit et al., 2004; Asahina et al., 2005; Chevaliez et al., 2007; Lutchman et al., 2007; Perelson and Layden, 2007; Cuevas et al., 2009; Dietz et al., 2013). A mutagenic activity of ribavirin has been documented in cell culture with HCV and subgenomic replicons (Contreras et al., 2002; Zhou et al., 2003; Kanda et al., 2004; Ortega-Prieto et al., 2013), albeit with exceptions

(Kato et al., 2005; Mori et al., 2011). The main problem to interpret the mechanism of anti-HCV activity of ribavirin *in vivo* is that this antiviral agent can act through several nonexclusive mechanisms, including: (i) immunomodulatory activity with enhancement of the Th1 antiviral response (Hultgren et al., 1998; Ning et al., 1998); (ii) upregulation of expression of genes related to IFN signaling pathways (Zhang et al., 2003; Feld et al., 2007); (iii) depletion of intracellular guanosine-5′-triphosphate (GTP) levels associated with the inhibition of inosine-monophosphate dehydrogenase (IMPDH, the enzyme that converts inosine-monophosphate into xanthosine-monophosphate in the GTP biosynthesis pathway) by ribavirin-monophosphate (RMP) (Streeter et al., 1973); (iv) inhibition of mRNA cap formation (Goswami et al., 1979); (v) inhibition of viral polymerases, independent of a mutagenic activity (Eriksson et al., 1977; Wray et al., 1985; Toltzis et al., 1988; Fernandez-Larsson et al., 1989; Maag et al., 2001; Bougie and Bisaillon, 2003); (vi) lethal mutagenesis, as evidenced with several RNA viruses (some of the studies are listed in Table 9.1), but not with other viruses (Leyssen et al., 2005, 2006; Kim and Lee, 2013). As general reviews of the antiviral properties of ribavirin, see (Snell, 2001; Graci and Cameron, 2002; Parker, 2005; Beaucourt and Vignuzzi, 2014).

It is not easy to separate an inhibitory from a mutagenic activity exerted by mutagenic base or nucleotide analogs. Inhibition may be a consequence of mutagenesis or inhibition and mutagenesis may have two totally separate modes of action. In the case of the antiviral activity of FU against FMDV, it was possible to show inhibition of the uridylylation of primer protein VPg (the initial step of picornavirus RNA synthesis) by 5-fluorouridine-triphosphate (FUTP), in addition to a mutagenic activity of FUTP during RNA elongation (Agudo et al., 2008). In the case of ribavirin, the situation is more complex since the involvement of many cellular functions in HCV replication (in reality in the replication of any virus), does not allow excluding the participation of any of the six mechanisms listed in the previous paragraph in its anti-HCV activity. In particular, it would be interesting to reexamine the evidence for inhibition of viral polymerases to ascertain that the inhibition is independent of a mutagenic activity.

In my group we favor lethal mutagenesis as being part of the antiviral activity of ribavirin against HCV, without excluding the participation of other mechanisms *in vivo*. The main arguments on which our preference is based are: in the study by (Dietz et al., 2013) that involved next generation sequencing (NGS) analyses of HCV from patients subjected to ribavirin monotherapy, the virus showed the mutational bias expected from ribavirin mutagenesis. A second argument is the consistent ribavirin mutagenesis of HCV observed in cell culture that could not be accounted for by GTP depletion (see Ortega-Prieto et al., 2013 and references therein). It has been suggested that in cell culture experiments the concentrations of ribavirin used are not attainable *in vivo*. Until measurements of the concentration of ribavirin nucleotides at the HCV replication complexes are available, it cannot be concluded that ribavirin concentrations *in vivo* are incompatible with mutagenesis; also, a broad range of ribavirin concentration in human serum have been reported during treatment (discussion and references in Ortega-Prieto et al., 2013). Furthermore, there are several reasons to miss a mutagenic activity *in vivo* with current analytical procedures. Ribavirin produced transient expansions followed by compression of mutant spectrum complexity in model studies with FMDV in cell culture (Ojosnegros et al., 2008; Perales et al., 2011b) (Figure 9.9). A possible interpretation is that when the mutational load due to ribavirin mutagenesis surpasses some critical value, increasingly defective genomes cease to contribute to progeny, therefore, resulting in mutant spectrum compression. The compression can be viewed as a mutagenesis-mediated bottleneck-associated reduction of complexity. Additional variations in FMDV mutant spectrum complexity were observed upon other mutagenic treatments and their interruption (discussed in Ojosnegros et al., 2008).

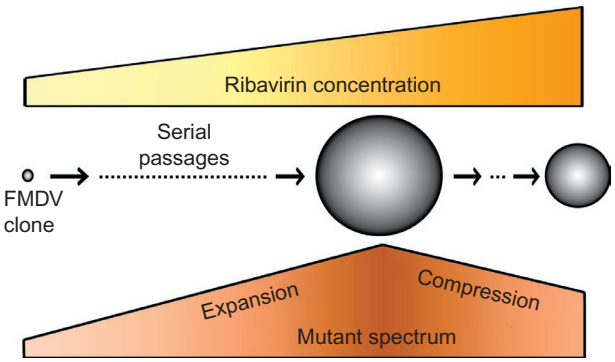

FIGURE 9.9

Expansion and compression of a FMDV mutant spectrum following ribavirin mutagenesis. A biological clone of FMDV (left) was subjected to serial passages in the presence of increasing concentrations of ribavirin (200 up to 5000 μM). The size of the three spheres quantifies in an approximate manner the complexity of the mutant spectrum, following the average distance parameter used to quantify genome subpopulations in the partition analysis of quasispecies (PAQ) clustering procedure of Baccam et al. (2001). The amplitude of the mutant spectrum increased first, but then it was compressed to a significant extent. The scheme is based on data reported in (Ojosnegros et al., 2008) and possible interpretations and implications are discussed in the text.

These model studies render unsurprising that no consistent expansions of HCV mutant spectra have been observed in patients subjected to ribavirin-based treatments or in chronically infected, untreated patients (compare, e.g. Farci et al., 2000; Duffy et al., 2002; Sullivan et al., 2007; Ramachandran et al., 2011; Palmer et al., 2014). Thus, even during ribavirin treatment alteration of mutant spectrum complexity may be missed depending on the time after treatment onset at which the viral sample is obtained. Also, it is necessary to quantify the amount of viral RNA to ensure that the sequence analyses either by standard molecular cloning-Sanger sequencing or NGS provide a faithful representation of the biological sample (see Section 3.6.4 in Chapter 3). Biases in the quantification of mutant spectrum complexity due to limitation in the initial amount of viral nucleic acid cannot be excluded unless sufficient methodological detail is described. Finally, in our view it is unlikely that a mutagenic activity readily observed in cell culture may be totally absent and bear no relationship with the activity of ribavirin *in vivo*, even though other mechanisms of ribavirin action can also be involved.

The question of participation of lethal mutagenesis in the *in vivo* antiviral activity of ribavirin, favipiravir, and other antiviral nucleotide analogs under development is expected to be solved soon, given the increasing application of NGS methodologies. If the answer were positive it would mean that inadvertently lethal mutagenesis might have been the mode of action of broad-spectrum antiviral agents used for decades, and traditionally considered standard nonmutagenic inhibitors.

9.6 VIRUS RESISTANCE TO MUTAGENIC AGENTS: MULTIPLE MECHANISMS AND EVIDENCE OF ABORTIVE ESCAPE PATHWAYS

It took more than one decade of use of ribavirin to obtain the first resistant mutants using Sindbis virus and mycophenolic acid (a nonmutagenic inhibitor of IMPDH); the selected mycophenolic acid-resistant

mutants displayed cross-resistance to ribavirin (Scheidel et al., 1987; Scheidel and Stollar, 1991). A decade later the first ribavirin-resistant mutants of poliovirus selected in the laboratory (Pfeiffer and Kirkegaard, 2003), and of HCV from patients under ribavirin monotherapy were described (Young et al., 2003). Subsequent work has characterized viral mutants resistant to mutagenic agents, particularly ribavirin, FU, and 5-AZA-C (Pfeiffer and Kirkegaard, 2005b; Sierra et al., 2007; Arias et al., 2008; Agudo et al., 2010; Levi et al., 2010; Arribas et al., 2011; Feigelstock et al., 2011; Domingo-Calap et al., 2012; Sadeghipour et al., 2013; Zeng et al., 2013, 2014; among other studies). The major overall conclusion of these investigations is that viruses can develop resistance to mutagenic nucleotide analogs as they do to nonmutagenic inhibitors.

Several mechanisms of resistance to mutagenic nucleotide analogs have been described, and the major ones are listed in Box 9.3. They can be broadly divided into two main categories: modifications of the viral polymerase and modification of other proteins. The first substitution to be described that conferred ribavirin resistance was G64S in the PV polymerase (3D), that resulted in an increase of polymerase template-copying fidelity. Studies with PV harboring this mutation have been instrumental to show the relevance of mutant spectrum complexity in virus adaptability (Pfeiffer and Kirkegaard, 2005a; Vignuzzi et al., 2006) (see Section 2.6 in Chapter 2). These and other studies have established the important concept that to maintain an adequate fitness and a good survival probability, a virus population must keep its genome heterogeneity within a suitable range: too low a diversity impairs adaptability, and too high a diversity may approach the population to an extinction threshold (Smith et al., 2013; Smith and Denison, 2013; Zeng et al., 2014). Several studies have demonstrated an attenuation phenotype associated with low- or high-fidelity viral mutants (Borderia et al., 2016). In a clinical setting, maintaining mutant spectra of viruses within an optimal range is one of the predictors of viral survival and progression toward disease (Section 8.8 in Chapter 8).

BOX 9.3 MAIN MOLECULAR MECHANISMS OF RESISTANCE TO MUTAGENIC NUCLEOTIDE ANALOGS

- Amino acid substitutions in the viral polymerase:
  Substitutions that increase the general copying fidelity of the enzyme.
  Substitutions that specifically limit the incorporation of the mutagenic nucleotide.
  Substitutions that modulate the relative incorporation of the standard nucleotides.
- Amino acid substitutions in other viral proteins:
  Substitutions in nonstructural viral proteins that participate in viral replication or modify polymerase fidelity.
- Combinations of some of these mechanisms.
- Fitness-enhancing mutations (unrelated to resistance *per se*) can contribute to the expression and stability of the resistance trait.

(Most virological studies have involved base or nucleoside analogs rather than nucleoside-triphosphates. It is assumed that at least for the mechanisms described to date, base and nucleosides are converted into the nucleoside-triphosphate derivatives which are responsible for the mutagenic activity. See text for references).

9.6.1 UNPREDICTABLE EFFECTS OF SOME POLYMERASE SUBSTITUTIONS

The same amino acid substitution in the polymerase of related viruses may have a totally different phenotypic effect. The ribavirin resistance, fidelity-enhancing PV substitution G64S in 3D was obtained independently in two different laboratories (Pfeiffer and Kirkegaard, 2003; Castro et al., 2005). This suggested that PV may have a very restricted number of mutations to attain ribavirin resistance, despite ample evidence that viruses generally display alternative pathways toward resistance to nonmutagenic inhibitors (Section 8.4.5 in Chapter 8). When FMDV whose polymerase is closely related to that of PV (Ferrer-Orta et al., 2004) was passaged in the presence of ribavirin, replacement G62S (the one equivalent to G64S in PV) was not selected. Instead, the 3D substitution selected was M296I (Sierra et al., 2007). A mutant FMDV encoding G62S in 3D that was constructed by site-directed mutagenesis, displayed a strong selective disadvantage relative to the standard virus, that was partially compensated by the presence of M296I. The mutant reverted upon passage in cell culture while FMDV with substitution M296I in 3D was stable (Ferrer-Orta et al., 2010). The comparison of the enzymological properties of the FMDV polymerase (3D) with either G62S, M296I, or both indicated that G62S impairs RNA binding, RNA polymerization, and the incorporation of RMP into RNA. Therefore, despite G62S being of potential benefit for the replication of FMDV in the presence of ribavirin, its selection would be abortive. Despite the sites of substitution G62S and M296I being separated by 13.1 angstroms, a network of interactions allowed a cross influence between the two sites, with an effect on the catalytic domain of the enzyme (Ferrer-Orta et al., 2010). Thus, due to distance effects and the subtle, sequence-dependent network connections among residues within the polymerase molecule, the same amino acid replacement may have disparate and unpredictable effects on the behavior of closely related polymerases.

9.6.2 POLYMERASE FIDELITY AND MODULATION OF NUCLEOTIDE INCORPORATION

Substitution M296I in 3D of FMDV offers an example of mutagen resistance that limited the incorporation of RMP in the viral RNA without a significant alteration of the general template-copying fidelity of the enzyme (Sierra et al., 2007; Arias et al., 2008) (Box 9.3). Fitness of the mutant FMDV relative to the standard virus was 3.8 in the presence of 800 μM ribavirin, and 0.5 in the absence of ribavirin [these values represent a selective strength of 7.6 for substitution M296I ($f_{+\text{Ribavirin}}/f_{-\text{Ribavirin}}$), calculated as described in Section 8.4.4 of Chapter 8].

The selective strength conferred by substitution M296I was not sufficient to maintain this substitution in 3D as the only one for the virus to respond to higher ribavirin concentrations. Passage of the 3D M296I mutant virus in the presence of larger concentrations of ribavirin, resulted in selection of two additional substitutions, P44S and P169S, to yield a virus with the triple substitution P44S, P169S, and M296I in 3D; this triple mutant was termed SSI (Agudo et al., 2010). The selective strength of the triple mutant measured in the presence of 800 μM ribavirin was 18.3, 2.4-fold higher that the selective strength conferred by M296I alone. Significantly, the most salient biochemical feature displayed by the mutant polymerase was that P44S restricted the incorporation of RMP more strongly opposite C than opposite U in a number of *in vitro* incorporation assays. As a consequence, during replication in the presence of ribavirin, mutant SSI could maintain a balance of the different transition types [measured as the ratio of (G→A)+(C→U) to (A→G)+(U→C) mutations] typical of the standard virus when replicating in the absence of mutagens (Agudo et al., 2010) (see Figure 9.8 for the ribavirin-mediated mutational pathways). The modulation mechanism associated with 3D substitution P44S in FMDV is one of the mutagen resistance mechanisms listed in Box 9.3. Modulation of transition types

allows the virus to maintain its typical mutant spectrum complexity with its corresponding adaptability. Interestingly, substitution P169S contributed a selective advantage only in the presence of very high (5000 μM) ribavirin concentrations, once the transition-modulating phenotype through P44S had already been acquired. Box 9.3 lists also as mutations contributing to mutagen escape those that increase fitness without being *bona fide* resistance mutations. Comparison of the fitness-enhancing mutations described for antiviral resistance (Chapter 8) and the effect of P169S in 3D of FMDV suggests that such mutations can be divided in two classes: those that increase fitness generally and those that do so only in the presence of mutagen and even in the presence of some range of mutagen concentrations (Agudo et al., 2010).

The analyses of mutant spectra produced by FMDV with the standard and SSI 3D in the absence and presence of ribavirin suggest that a bias in favor of G→A and C→U is detrimental to the virus because it is associated with an increase of nonsynonymous mutations, and probably also of defective genomes, as evidenced by the presence of a stop codon in one of the clones of the mutagenized standard virus population (Agudo et al., 2010).

The three-dimensional structures of the ribavirin-resistant polymerases revealed alterations in the N-terminal region of 3D (Agudo et al., 2010), affecting sites that belong to a nuclear localization signal (NLS) present in FMDV 3D (Sanchez-Aparicio et al., 2013). Amino acid substitutions within the NLS that diminished the transport to the nucleus of 3D and 3D3C (a functional precursor intermediate of 3D and the protease 3C) modified also the template binding and nucleotide recognition properties of 3D (Ferrer-Orta et al., 2015). Interestingly, some replacements within the NLS increased the incorporation of RMP relative to standard substrates, suggesting that structural alterations in viral polymerases may enhance the vulnerability of viruses to nucleotide analogs. Despite uncertainties repeatedly exposed in this book (dependence of enzyme behavior on the protein and template sequence context, behavior modifications due to introduction of additional amino acid substitutions, etc.) it can be envisaged that through structure-based designs, new drugs could be found that enhance the incorporation of mutagenic nucleotides and contribute to new antiviral combinations. The results on the effect of amino acid substitutions within the NLS of FMDV 3D emphasize the multifunctional nature of this viral polymerase, in line with the recognized multifunctionality of many viral proteins, a feature that increases vulnerability to lethal mutagenesis.

The comparison of the several amino acid and groups of amino acid substitutions that in 3D of FMDV can be involved in ribavirin resistance (Sierra et al., 2007; Agudo et al., 2010; Zeng et al., 2014; Ferrer-Orta et al., 2015) suggests that there are multiple, in some cases even independent, evolutionary pathways for a virus to achieve resistance to mutagenic agents, as there are multiple pathways toward resistance to standard, nonmutagenic inhibitors. In the case of PV, different ribavirin resistance substitutions would be expected if independent viral lineages were subjected to a range of ribavirin concentrations.

Furthermore, the viral polymerases are not the only determinants of template-copying fidelity. The viral replicative machineries consist of a complex of several viral and host proteins, and it has been shown that proteins other than the polymerase can also contribute to polymerase fidelity and resistance to mutagenic agents (Box 9.3). The possibility that the read through protein of bacteriophage Qβ may contribute to 5-AZA-C resistance was suggested by a study of E. Lázaro and colleagues (Arribas et al., 2011), although limited amino acid substitutions in the viral replicase can confer resistance (Cabanillas et al., 2014). The small, nonenzymatic coronavirus nonstructural protein 10 is involved in maintaining the replication fidelity of the virus (Smith et al., 2015). Two substitutions in protein NS5A of an HCV replicon conferred low-level resistance to ribavirin (Pfeiffer and Kirkegaard, 2005b). In the

case of the DNA bacteriophage ϕX174 that does not encode its own polymerase, FU resistance was achieved through substitutions in the virus-coded lysis protein (Pereira-Gomez and Sanjuan, 2014). It was proposed that delayed cell lysis, increase in the amount of progeny virus per cell, and limitation of the number of infectious cycles reduced the chances of mutagenesis. Therefore, current evidence anticipates multiple adaptive mechanisms of virus resistance to mutagenic nucleotides, not necessarily confined to the polymerase as documented for standard inhibitors. There is, however, an interesting possibility of an initial advantage of mutagenic agents over standard inhibitors to impede selection of resistant mutants. Before justifying this suggestion, current therapeutic alternatives based on lethal mutagenesis are discussed.

9.7 VIRUS EXTINCTION AS THE OUTCOME OF REPLACEMENT OF VIRUS SUBPOPULATIONS: TEMPO AND MODE OF MUTATION ACQUISITION

Some general considerations derived from the observations described in previous sections are worth commenting. The extinction of a virus is not only a consequence of the reduction of viral load (with insufficient R_0 value to ensure cell-to-cell transmission to sustain the infection; see Section 7.2 in Chapter 7 for the concept of R_0), or of a simple movement in sequence space, or even of an increase of mutational load in replicating genomes. It is the result of a combination of several influences under specific circumstances of the kinetics of accumulation of mutations in viral genomes. We consider these points in turn.

Studies by N. Pariente, C. Perales, and colleagues showed that a given reduction in viral load achieved through mutagenesis was sufficient to drive FMDV toward extinction, but that when the same reduction was achieved through inhibition, it did not lead to virus extinction (Pariente et al., 2001, 2003, 2005; Perales et al., 2011a). The main difference is that mutagenesis is a dynamic process of genome variation and reduction of viral load, while inhibition is basically a static process regarding incorporation of mutations. Only when a decrease in viral load has already taken place, the virus becomes more vulnerable to mutagenesis (Sierra et al., 2000).

Movements in sequence space are the norm during virus replication and evolution, independently of being subjected to increased mutagenesis or not. An A, U-rich genome subpopulation that became dominant upon passage of the virus in the presence of ribavirin existed also in the standard viral population, albeit at a lower level (Perales et al., 2011b). That is, the main effect of ribavirin was to shift the dominant part of the mutant spectrum into an A, U-rich region. This shift may increase the presence of defective genomes, including defectors (Section 9.3.1). We encounter here again one of the main departures introduced by quasispecies in the understanding of virus evolution: replacement of some genome subpopulations by others. In the case of extinction, the movement consists in the replacement of standard subpopulations by others lying in unfavorable portions of sequence space. Expressed in another manner: mutagenesis forces an uncontrolled, not a fitness gradient-mediated, relocalization in sequence space.

The mutational load, calculated as the number of mutations incorporated in an individual genome is not by itself a predictor of survival or extinction. It depends on the location of the mutations. Accumulation of mutations may reduce fitness and robustness to the effect of additional mutations (Section 6.7.1 in Chapter 6) but still allow replicative competence. A FMDV clone subjected to multiple plaque-to-plaque transfers accumulates mutations at a rate of 0.1-0.25 mutations per passage, and viable genomes can accumulate a number of mutations equivalent to a mutation frequency of

10^{-2} substitutions per nucleotide (Escarmís et al., 2008). A 10-fold lower mutation frequency is sufficient to extinguish the same viral clone upon passage in the presence of a mutagenic agent. The tempo and mode of accumulation of mutations is the determinant factor. E. Lázaro and colleagues showed that 5-AZA-C enhanced Qβ replication during plaque development, but drove the virus to extinction during growth in liquid culture medium, unless a mutagen-resistant Qβ was selected (Cases-Gonzalez et al., 2008; Arribas et al., 2011). The experimental design in the plaque transfers is such that at each transfer a viral particle capable of developing a visible plaque on the cell monolayer is rescued no matter how many companions are left behind (extinguished). The applied selection is powerful. In contrast, during mutagenesis without intervening plaque isolations, no means to recover viable minority survivors are included in the design: if a rare potential survivor with a cluster of mutations arises transiently, it will soon perish due to the next rounds of mutagenesis, or will be suppressed by the surrounding mutant spectrum. Thus, even if beneficial mutations are more likely to arise in low-fitness genomes, their contribution to survival is minimal under a mutagenic environment.

A critical point for prediction of virus extinction is how close to an error (or extinction) threshold virus replication takes place. The evidence is that DNA genomes whose replication is catalyzed by a high-fidelity DNA polymerase may resist transient increases of mutation rate and even benefit from them (Cupples and Miller, 1989; Solé and Deisboeck, 2004; Springman et al., 2010). Thus, viral extinction by enhanced mutagenesis is conditioned by several factors related mainly to basal replicative parameters of the virus, and the kinetics of mutagenesis.

9.8 THE INTERPLAY BETWEEN INHIBITORS AND MUTAGENIC AGENTS IN VIRAL POPULATIONS: SEQUENTIAL VERSUS COMBINATION TREATMENTS

As a test of the paradigm of the general advantage of combination therapies over monotherapy, initial experiments were designed to study the efficacy of a combination of a mutagenic agent and an antiviral inhibitor. The results documented that extinction of high-fitness FMDV or HIV-1 could be achieved by a combination of a mutagenic agent and a nonmutagenic inhibitor, but not with the mutagenic agent alone (Pariente et al., 2001, 2005; Tapia et al., 2005). Despite being an expected result, additional experiments pursued by C. Perales with FMDV demonstrated that the response of the virus to the combined action of an inhibitor and a mutagenic agent is a bit more complex than initially thought. The critical comparative experiment was performed by C. Perales, and it is shown in Figure 9.10. The production of infectivity and viral RNA was measured in the course of viral passages in the presence of the inhibitor guanidinium chloride alone, or ribavirin alone, or a combination of guanidinium chloride and ribavirin, or with an initial passage in the presence of guanidinium chloride, followed by passages in the presence of ribavirin alone (Figure 9.10a). The results of quantification of FMDV progeny and a sensitive RT-PCR test of virus extinction, documented that the design consisting of administering guanidine alone first, followed by additional passages in the presence of ribavirin alone was the most effective to reduce progeny production and to drive the virus toward extinction (Figure 9.10b–e) (Perales et al., 2009b). S. Manrubia developed a theoretical model whose main parameters were the concentration of standard and defective viruses sensitive and resistant to the inhibitor, the viral mutation rate, the rate of generation of inhibitor-resistant mutants, the number of standard and defective progeny genomes, and the number of infected cells and of infectious cycles per cell. The model (described in

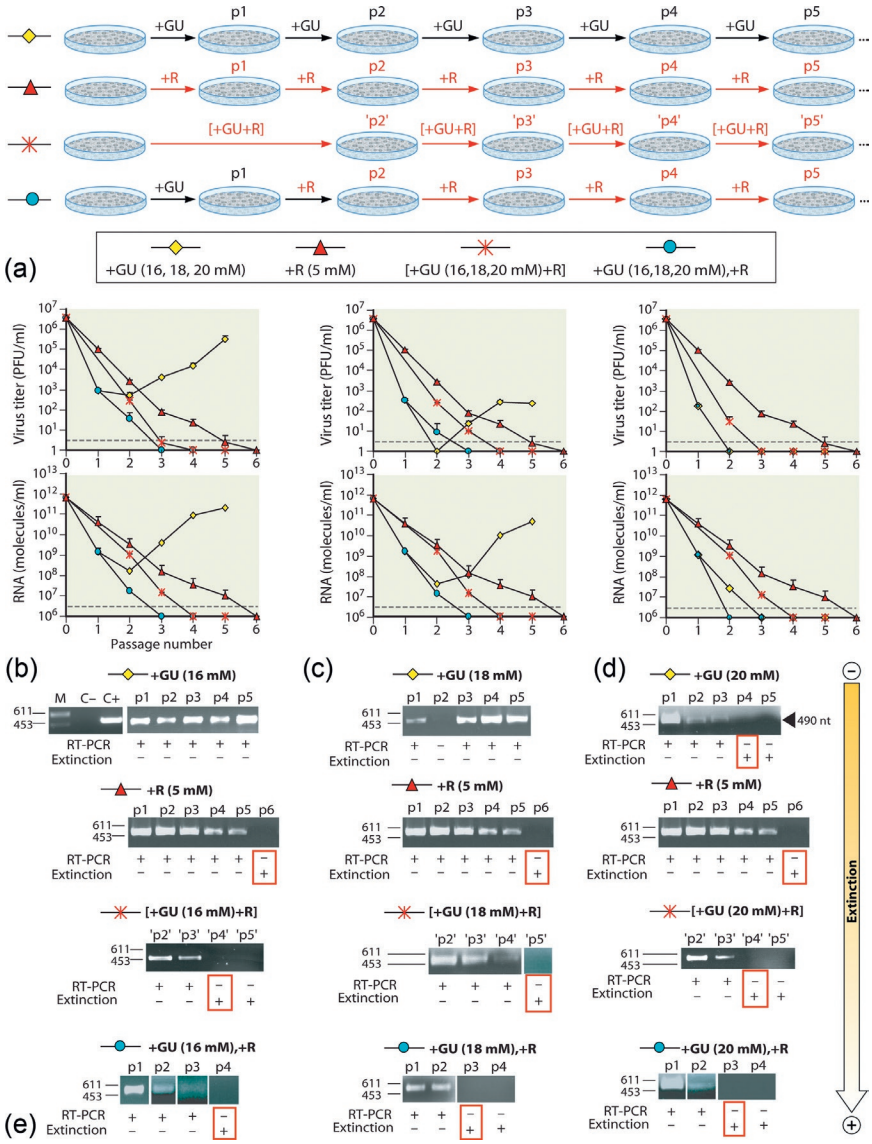

FIGURE 9.10

Alternative antiviral designs using an inhibitor and a mutagenic agent. This model study was carried out with FMDV replicating in BHK-21 cells. The inhibitor used was guanidine hydrochloride (GU), and the mutagen was ribavirin (R). (a) The four types of serial passages (p indicates passage number) are, from top to bottom: passages in the presence of guanidine alone (yellow diamond); passages in the presence of ribavirin (R) alone (red triangle); passages in the presence of a mixture of GU and R (denoted as [+GU+R]; red star; passage number is in quotes because the first passage with the mixture of GU and R was considered equivalent to the second passage in the presence of a single drug); finally, a first passage in the presence of GU, followed by four passages in the presence of R (blue circle). (b), (c), (d) Virus titer and viral RNA level in the course of six passages in the presence of 5 mM R and 16, 18, 20 mM GU in (b), (c), (d), respectively, with passage regimen code indicated in the box. Note that the highest reductions of FMDV progeny production are achieved using the sequential GU-R protocol (blue circles) and that infectivity and viral RNA are lost earlier at the highest GU concentration tested. (e) A highly sensitive RT-PCR amplification used as diagnostic of viral extinction confirmed the advantage of the sequential GU-ribavirin treatment. Symbols are as in (a).

Figure reproduced from Perales et al. (2009b) where additional experimental details can be found.

Perales et al., 2009b; Iranzo et al., 2011) explained the advantage of the sequential inhibitor-mutagen administration protocol over the corresponding combination. When an inhibitor and a mutagen are present together during viral replication, their joint influence on the viral RNA may jeopardize virus extinction. Some possible mechanisms of interaction between inhibitors and mutagenic agents are summarized in Box 9.4. The first of the points listed in Box 9.4 concerns us here: the presence of the mutagen may increase transiently the frequency of inhibitor-resistant mutants, therefore, favoring virus escape and treatment failure. A second mechanism by which the simultaneous presence of an inhibitor and mutagen can impede virus extinction is that in the presence of the inhibitor, the RNA defectors that are generated by mutagenesis of the viral RNA cannot interfere with viral replication. The reason is that defector replication is needed for lethal defection (Section 9.3.1), presumably because replication increases the amount of RNA that encodes *trans*-acting substituted proteins that are detrimental to the virus life cycle, and responsible for lethal defection.

The advantage of sequential inhibitor-mutagen administration has been evidenced also with LCMV, taking advantage of the double inhibitor and mutagenic character of ribavirin, that depends on its concentration (Moreno et al., 2012). The model that supports the advantage of sequential treatments predicts that such an advantage depends on set of replicative parameters of the virus and on the intensity of inhibition and mutagenesis (Iranzo et al., 2011; Perales et al., 2012). For each virus-host system, it will be necessary to carry out experiments to delimit the range of inhibitor and mutagen concentrations at which the sequential treatment is advantageous. The translation into clinical practice is of interest, but it will have the added complications derived from target compartmentalization and uneven inhibitor and mutagen concentrations in different compartments (Steinmeyer and Wilke, 2009). The model studies by S. Manrubia and J. Iranzo predict also that when therapy is based on the use of either two mutagenic agents or two inhibitors, a combination treatment is always preferred over the sequential. Only a few studies have investigated the consequences of using two mutagens together, with or without inhibitors (Perales et al., 2009a; Dapp et al., 2012). The availability of two or more virus-specific mutagen agents of different mutational preferences has the advantage that they will target different regions of sequence space (related to network connections discussed in Chapter 10), although the resulting mutational patterns may be difficult to interpret (Dapp et al., 2012). Also, a mutagen may extinguish a virus that has acquired resistance to another mutagen as in the extinction of a ribavirin-resistant FMDV mutant by FU (Perales et al., 2009a). According to the model, the worst option is to administer first a mutagen and then an inhibitor. Unless the mutagen achieves extreme reductions of viral load, it may generate an expanded mutant spectrum from which inhibitor-resistant mutants may be selected (Iranzo et al., 2011; Perales et al., 2012).

BOX 9.4 INTERACTIONS BETWEEN INHIBITORS AND MUTAGENIC NUCLEOTIDES THAT CAN AFFECT THE EFFICACY OF COMBINATION TREATMENTS

- A mutagen can increase the frequency of inhibitor-resistant mutants.
- An inhibitor can prevent replication of interfering mutants that contribute to lethal defection.
- The mutant spectrum can suppress inhibitor-resistant mutants that affect a *trans*-complementable protein.
- A mutagenized mutant spectrum can suppress high-fitness genomes.

See text for justification and references.

9.9 PROSPECTS FOR A CLINICAL APPLICATION OF LETHAL MUTAGENESIS

Lethal mutagenesis constitutes an example of how a fundamental theoretical concept initially unrelated to virology can unfold into a potential application in the form of antiviral designs. This transition is exemplified by M. Eigen, who established the basis of quasispecies theory (Eigen, 1971) and commented a paper on virus extinction by mutagenic nucleotides 30 years later! (Eigen, 2002). Can you imagine a grant application to work on quasispecies with the aim of controlling viral infections? Some readers will probably object immediately: nothing has been achieved yet with lethal mutagenesis at the clinical level. True, unless ribavirin has been clearing HCV with participation of mutagenesis (see Section 9.5). To be able to talk about the prospect of an application is sufficient to make the point about the relevance of basic research, and it is the prospect that we address here.

Box 9.5 lists advantages and limitations of lethal mutagenesis treatments as compared with standard, nonmutagenic inhibitors. Quantifications of mutagen-escape mutant frequencies have not been performed. The derivation of some of the mutants that have been studied (Section 9.6) required a gradual increase of mutagen concentration in the course of selective passages. The difficulty to isolate mutants from a mutagen-treated viral population may stem from the suppressive environment created by the mutagenesis itself that may preclude or delay dominance of the resistant mutant.

The possibility of sequential inhibitor-mutagen treatments (Section 9.8) is clinically relevant because such designs may diminish the severity of side effects by avoiding the simultaneous presence of two drugs in the treated patients. Shorter treatment duration of sequential treatments is also a possibility.

Some natural cellular mechanisms of defense against genetic parasites are based on producing an excess of mutations in the invader. In Chapter 2 (Section 2.7), APOBEC (*a*polipoprotein *B* mRNA

BOX 9.5 ADVANTAGES AND LIMITATIONS OF ANTIVIRAL TREATMENTS BASED ON LETHAL MUTAGENESIS

Advantages

- A possible high barrier to resistance.
- Its mechanism of action favors suppression of possible resistant variants by the mutagenized mutant spectrum within infected cells.
- Lethal mutagenesis may be included in sequential or combination designs with other classes of antiviral agents.
- Natural mechanisms of resistance to genetic parasites include lethal mutagenesis-like strategies: APOBEC, ADAR, RIP, etc.

Limitations

- The mutagenic activity of the agents must be virus specific. It cannot mutagenize cellular nucleic acids.
- Possible off-target effects are still poorly understood.
- The number of available antiviral mutagenic agents is restricted.
- Additional experiments with animal models and preclinical and clinical trials are needed prior to possible therapy implementation.
- Resistance of expert panels to encourage the exploration of unconventional antiviral approaches both for scientific and commercial reasons.

*e*diting *c*omplex) and ADAR (*a*denosine *d*eaminase *a*cting on double-stranded *R*NA) were discussed. They exert nucleic acid editing functions used by the cell that can be recruited as antiviral responses (Harris and Dudley, 2015). Editing is part of replication cycle of several viruses. It has been suggested that *Paramyxovirinae* might have evolved to possess a genome of polyhexameric length (known as the "rule of six") to avoid uncontrolled editing and error catastrophe of the virus (Kolakofsky et al., 2005). There are additional mutagenic-like activities that mimic lethal mutagenesis. One of them is termed RIP (*r*epeat-*i*nduced *p*oint mutations) that operates in some filamentous fungi to mutate genetic intruders, including transposable elements (Galagan and Selker, 2004; Clutterbuck, 2011; Braga et al., 2014; Amselem et al., 2015). Some experts regard as highly positive that an intended medical intervention resembles some natural process.

Box 9.5 lists also several limitations, some of which are obvious (need of specificity to mutagenize viral but not cellular nucleic acids, lack of information on off-target effects, and a necessity to explore treatment efficacy *in vivo*). It is likely that the number of virus-specific mutagenic agents will increase in coming decades. If their efficacy *in vivo* can be properly documented, expert panels may flexibilize their attitude toward these and other new antiviral approaches.

9.10 SOME ATYPICAL PROPOSALS

Decisions on the suitability of new treatments are mainly based on the three basic parameters CC_{50} (as a measure of toxicity), IC_{50} (as a measure of inhibitory potential), and the therapeutic index that they yield ($TI = CC_{50}/IC_{50}$) (explained in Section 8.4.3 of Chapter 8). There is an additional parameter that should also be considered: the spectrum of antiviral activity, regarding the number of unrelated viral pathogens that are effectively inhibited by the treatment. Although evidence is still lacking, the possibility that broad-spectrum in the above sense may predict a capacity to inhibit broad repertoires of quasispecies swarms is appealing. The broadness of antiviral activity is basically dependent on the mechanism of antiviral activity. In this chapter and in the preceding chapter, we have described two classes of broad-spectrum antiviral inhibitors: those that stimulate the innate immune response (notably inhibitors of pyrimidine biosynthesis) and mutagenic nucleotide analogs (notably ribavirin and favipiravir as the most relevant examples, likely to be followed by additional ones). Thus, it would be interesting to investigate the joint use of these two classes of compounds (stimulators of immune responses and lethal mutagens) in sequential or combination protocols of the type described in Section 9.8.

Drug repositioning may offer additional possibilities. F. Sobrino and colleagues demonstrated that valproic acid (2-propylpentanoic acid, VPA) displays a broad antiviral activity against many enveloped viruses (Martin-Acebes et al., 2011; Vazquez-Calvo et al., 2013). VPA is used to treat epilepsy and bipolar mania, among other disorders. It is listed among the essential medicines by the World Health Organization. Despite its antiviral potency being modest, its inclusion as part of combined therapies should be also considered in view of its broad-spectrum of activity. Triple, broad-spectrum drug combinations appear as an attractive possibility provided antagonistic interactions are avoided and side effects are tolerable.

There is a long way to go before lethal mutagenesis or other new designs based on the concepts listed in Box 9.1 can be applied to the treatment of viral disease. However, the hope is that the experience gained mainly with HIV-1 but also with other error-prone viruses, will favor explorations that regard the adaptive potential of viruses as the major challenge to be confronted.

9.11 OVERVIEW AND CONCLUDING REMARKS

It is uncertain whether the possibilities presented here as new trends in antiviral strategies will satisfy the demands of new paradigms to approach infectious disease (emphasized in the opening paragraphs of this chapter), or it will be necessary to wait for more innovative advance. The available possibilities have as a common trend that they respond to the quasispecies challenge, and fulfill requirements expressed repeatedly in this book: viral populations to be controlled should be denied any opportunity to replicate, because replication is synonymous with adaptation. Viruses have to be hit hard and as soon as possible both at the level of individual infections and at the epidemiological level. To the extent that new treatment designs can fulfill this requirement, they are worth being pursued.

Probably, combinations of broad-spectrum antiviral agents, irrespective of their nature (lethal mutagens, stimulators of the immune response, mixtures of highly neutralizing antibodies, or their combinations) are among the best options presently available. Despite the general preference for combination treatments, once a mutagenic agent enters the antiviral formulations, the possibility of sequential inhibitor-mutagen treatments should be considered. They may be as effective as the corresponding combinations and may alleviate the burden of side effects, one of the problems of current treatments.

Given the challenge of existing viral diseases and the emergent viral diseases unavoidably to come, what should not be done is to minimize the importance of the challenge and, as a consequence, withdraw the focus from the essential issue. Antiviral designs must be planned counting on quasispecies dynamics. Controversies about which theoretical models best describe extinction of viruses by lethal mutagenesis should be used to clarify antiviral mechanisms and find the adjustments for best efficacy. Controversies should not be presented as evidence against the real nature of dynamic quasispecies. With the application of deep sequencing to viral populations, the challenge has become dramatically evident. New antiviral targets, new drugs (mutagenic and nonmutagenic), and studies with animal models will hopefully contribute to reach the goal (see Summary Box).

SUMMARY BOX

- There is a need to develop new antiviral strategies to control viral pathogens characterized by quasispecies dynamics.
- Several new designs have been implemented or are under investigation. They aim at increasing the barrier to resistance or diminishing viral fitness.
- Lethal mutagenesis consists in virus extinction by excess mutations. Experimental evidence suggests that lethal mutagenesis is directly related to the error threshold concept of quasispecies theory.
- Mutagen-resistant viral mutants have been isolated, and their study has been instrumental to document the adaptive value conferred by a mutant spectrum of adequate complexity.
- Sequential inhibitor-mutagen treatments may have an advantage over the corresponding combination, depending on replicative parameters of the virus to be controlled and the inhibitory and mutagenic intensities.
- New treatments using broad-spectrum mutagenic and nonmutagenic drug combinations offer new prospects for the control of error-prone viruses.

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