Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

CHAPTER

8

Quasispecies dynamics in disease prevention and control

Abbreviations

Ab Antibody
AIDS Acquired Immuno Deficiency Syndrome
CC_{50} Cytotoxic Concentration 50
CTL Cytotoxic T Cell
DAA direct-acting antiviral
FMDV Foot-and-Mouth Disease Virus
FU 5-Fluorouracil
HAV Hepatitis A Virus
HBV Hepatitis B Virus
HCV Hepatitis C Virus
HIV-1 Human Immunodeficiency Virus Type 1
IC_{50} Inhibitory Concentration 50
IFN-α Interferon-alpha
IV Influenza Virus
MAb Monoclonal Antibody
MOI Multiplicity of Infection
NNRTIs Nonnucleoside Reverse Transcriptase Inhibitors
NRTIs Nucleoside/nucleotide Reverse Transcriptase Inhibitors
RT Reverse Transcriptase
TI Therapeutic Index

8.1 Medical interventions as selective constraints

Medical interventions have dramatically increased over the last century, and in the case of infectious diseases, the discovery and development of antibiotics and antiviral agents have represented a very powerful external selective constraint imposed upon replicating microbes. Hundreds of antiviral agents have been developed since the second half of the 20th century, and viruses can generally find evolutionary pathways to continue replication in their presence. The same is true of antibiotics and replication of bacteria. The belief that bacterial diseases were on their way toward extinction was quite widespread in the middle of the 20th century. Sir M. Burnet wrote in his 1966 textbook: "And since bacterial infections are, with unimportant exceptions, amenable to treatment with one or other of the new drugs, our real problems are likely to be concerned with virus diseases" (Burnet, 1966). In 1932, a prominent Spanish medical doctor, G. Marañón, declared: "In the year 2000 cancer will be a historical disease. Infections will be almost entirely absent as a cause of mortality." (On a personal note, when I joined the University of California, Irvine in 1969, to work as postdoctoral student with R.C. Warner, I attended some biology courses in which the teachers expressed to students that infectious diseases would disappear in a few decades as a consequence of the use of antibiotics and antiviral agents). Predictions in science tend to fail.

The optimistic view was not unanimous. A. Fleming, the discoverer of penicillin, recognized the adaptive capacity of bacteria and suggested

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that bacteria would inevitably find ways of resisting the damage to them caused by antimicrobial drugs [quoted from the document "New antimicrobial drugs" from the European Academies Science Advisory Council, November 2014 (www.easac.eu); see also Chapter 10]. Furthermore, there was early evidence of selection of *Mycobacterium tuberculosis* mutants resistant to streptomycin (Mitchison, 1950). Antibiotic resistance in bacteria has similarities and differences with antiviral resistance in viruses, and they are compared in Chapter 10.

We are now very aware that one of the major problems in antiviral therapy is the nearly systematic selection of drug-resistant virus mutants, which is often associated with treatment failure. Other external influences, such as vaccination or immunotherapy, particularly using monoclonal antibodies, can also evoke the selection of viral subpopulations capable of replicating in the presence of those components inherent to an immune response. Thus, selective constraints intended to limit RNA virus replication meet with the broad and dynamic repertoire of variants ingrained in quasispecies dynamics. Two space-time levels of the effects of drugs or vaccines are distinguished in coming sections: (i) short-term consequences for the individual in the form of treatment or vaccination failure and (ii) long-term consequences at the population level in the field, or vaccine-driven evolution of the antigenic properties of viruses.

There are other medical interventions that may alter virus survival. Individuals who are immunocompromised as a consequence of treatment after organ transplantation or those subjected to anticancer chemotherapy become particularly vulnerable to viral infections. Enhanced viral replication can favor pathological manifestations in the affected individual as well as the spread of a large number of viruses into the environment, with consequences for the emergence and reemergence of viral disease (Section 7.7 in Chapter 7).

8.2 Different manifestations of virus evolution in the prevention and treatment of viral disease

Viral diseases are an important burden for human health and agriculture (Bloom and Lambert, 2003). Virus evolution, through the basic mechanisms exposed in previous chapters, can influence the two major strategies to combat viral infections: prevention by vaccination and treatment by antiviral inhibitors. For the design of new antiviral vaccines, a critical issue is the diversity displayed in the field by the virus to be controlled. The natural evolution of the virus may result in the circulation of one major antigenic type or the cocirculation of multiple antigenic forms. The vaccine composition (independently of the type of vaccine; see Section 8.3.1) must match the antigenic composition of the virus to be controlled. Hepatitis A virus (HAV) circulates as a single serotype, while foot-and-mouth disease virus (FMDV) circulates as seven serotypes and diverse subtypes, and the antigenic types are unevenly distributed in different geographical locations. A monovalent vaccine made of the prevailing antigenic type of HAV should be sufficient to confer protection, while a multivalent vaccine composed of several types or subtypes is required to confer protection against FMDV, and the antigenic composition of the vaccine should match the circulating viruses in each geographical region. This is why antiFMD vaccines of different compositions are used in different world areas at a given time, and vaccine composition must be periodically updated to maintain its efficacy. Thus, one effect of virus evolution relevant to vaccine design derives from the necessity to prepare a vaccine that mirrors the antigenic composition of the virus to be controlled. In the case of live-attenuated antiviral vaccines, the evolution of the vaccine virus while it replicates in the vaccinee is a risk factor to produce virulent derivatives.

The invasion of a susceptible host by a virus and the ensuing viral replication can be regarded as a step-wise process during which the virus must adapt to a series of selective pressures presented by the host, notably the immune response. The outcome can be either viral clearance (elimination of the infection) or virus survival and progression toward an acute or a persistent infection. Administration of antiviral agents is an additional selective constraint that limits viral replication. Evolutionary mechanisms may either succeed in the selection of mutants resistant to the antiviral agent that will permit the infection to continue or fail in sustaining the infection, resulting in the clearing of the virus from the organism.

Treatment planning, one of the aims of the new antiviral pharmacological interventions, based on information of viral genomic sequences present in each infected patient, has parallels with vaccine composition design. For vaccines, the information comes from the analyses of antigenic composition of circulating viruses, and for antiviral agents, the information comes from the mutant spectrum composition of the virus to be controlled in the infected patient.

8.3 Antiviral vaccines and the adaptive potential of viruses

World-wide vaccination campaigns made possible the eradication of human smallpox [with the official declaration by the World Health Organization (WHO) in 1980] and animal rinderpest [with the official declaration by the World Organization for Animal Health, Office International des Epizooties (OIE) in 2011]. The number of new cases has decreased as a result of vaccination programs against several viral diseases, including measles or hepatitis B (Bloom and Lambert, 2003), and substantial progress has been made toward the eradication of poliomyelitis (Chumakov and Kew, 2010; Himman, 2017). Only the deliberate decision not to vaccinate (for religious reasons or misinformation campaigns) or lack of vaccine accessibility (for socioeconomic circumstances) jeopardizes vaccine efficacy. These facts demonstrate that at least some viral diseases can be controlled on a global basis by vaccination, an unprecedented achievement of human and animal health.

Despite the huge economic investment, however, there are important viral diseases such as acquired immunodeficiency syndrome (AIDS), hepatitis C, or viral hemorrhagic fevers for which no effective vaccines are available. For some diseases such as human influenza or animal FMD, vaccines are accessible, but they require periodic updating to approximate the antigenic composition of the vaccine to that of the circulating virus (Section 8.2). In the case of influenza virus (IV), a major change in antigenic composition can occur through antigenic shift, in which the virus acquires new hemagglutinin and neuraminidase genes by genome segment reassortment (Section 7.4 in Chapter 7), with the first evidence obtained by G. Laver as early as 1971 (for the early history of influenza, its causative virus, and vaccine designs, see Beveridge, 1977; Kilbourne, 1987). Antigenic variation of viruses, whatever the mechanism might be, can affect vaccine efficacy and in some cases, the extreme rapid intra and interhost evolution of a virus may render an effective vaccine unfeasible at least with the current tools of vaccinology. $CD8^+$ T cell responses may act soon after infection and promote the selection of escape mutants (Bull et al., 2015). The difficulties for the control of virus disease derived from the adaptive potential of viruses (Domingo, 1989; Domingo and Holland, 1992; Bailey et al., 2004; Hamelaar et al., 2019) require the judicious application of existing tools and innovative approaches that are still in their infancy.

8.3.1 Some requirements for the design of vaccines to control highly variable viruses

A first basic requisite for the preparation of a vaccine against a viral agent is the understanding

of the immune response evoked by the virus when it infects the organism to be protected (activation of B and T lymphocytes for antibody production, cellular responses, and generation of memory cells) and correlates of protection (Bloom and Lambert, 2003; Van Regenmortel, 2012; Hagan et al., 2015; Cunningham et al., 2016; Rolland, 2019). Despite social pressure to rapidly obtain a vaccine, for each virus-host system, well-designed experiments are necessary to try to establish the determinants of protection, which is not a simple issue. The discussions in coming paragraphs are focused on the relevance of virus evolution in vaccine efficacy, irrespective of the type of protection afforded by the vaccine. What we term "protection" may mean the total absence of replication of the infecting virus (termed "sterilizing" immunity) or absence of disease manifestations despite infection and virus replication. As a general initial statement, which is widely accepted by vaccinologists, a vaccine is likely to be effective when it evokes an immune response that is similar to the response elicited by the authentic viral pathogen when it produces disease successfully overcome by the infected organism (Evans and Kaslow, 1997; Bloom and Lambert, 2003). We refer to this as the basic principle of vaccinology. When infection by an antigenically constant virus produces lifelong immunity (i.e., measles virus infection), a vaccine is likely to evoke long-lasting protection. In contrast, if a patient cured of a virus can be reinfected by the same (or a closely related) virus (i.e., hepatitis C virus infection) a vaccine—at least one prepared by standard methodology—is unlikely to evoke protection.

Some points to be considered in the design of antiviral vaccines are listed in Box 8.1. They are intended to minimize the selection of vaccine-escape mutants and favor the success of vaccination campaigns. Some of the recommendations deserve further comment. First, a basic knowledge of virus evolutionary dynamics and how it affects virus antigenic stability (or lack of) is essential. The fact that a methodology is available (i.e., vectors that can express large amounts of

BOX 8.1

VACCINE DESIGNS AND VACCINATION STRATEGIES FOR ANTIGENICALLY VARIABLE VIRUSES

- Prior to the planning of a vaccine strategy, it is essential to review what is known about genetic and antigenic variation of the virus to be controlled (whether it is a DNA or RNA virus displaying high- or low-fidelity replication, antigenic diversity in the field, location of B- and T-cell epitopes, etc.).
- Carry out research to understand the correlates of protection.
- In keeping with the basic principle of vaccinology, from the point of view of inducing a protective response the preferred order of vaccine types is as follows: live attenuated > whole virus inactivated = empty viral particles > multiple immunogenic viral proteins > a single immunogenic viral protein > mixtures of synthetic peptides, dendrimeric scaffolds, peptide arrays > a single synthetic peptide.
- International vaccination programs should be carried out as quickly as possible.
- Programs to update the antigenic composition of vaccines should be implemented.

Based on Domingo and Holland (1992).

antigens displaying good immunogenicity) does not guarantee vaccine efficacy, and even less if correlates of protection are not understood. The order of efficacy of different vaccine designs proposed in Box 8.1 is justified both by the basic principle of vaccinology and by the mechanisms of selection of antibody (Ab)- and cytotoxic T-cell (CTL)-escape mutants by viruses. Single amino acid substitutions at B- and T-cell epitopes in viral proteins are often sufficient to elude neutralization by the corresponding cognate-specific antibody or to escape recognition by a clonal CTL population. For many viruses, the frequency of monoclonal antibody-escape mutants has been measured in 10^{-4} to 10^{-6}, even in clonal populations obtained under controlled laboratory conditions and that have undergone a limited number of replication rounds (Section 7.4.2 in Chapter 7). Generation of immune-escape variants can result in lack of vaccine efficacy, contribute to viral persistence (Pircher et al., 1990; Weidt et al., 1995; Ciurea et al., 2000, 2001; Richman et al., 2003; Pawlotsky, 2006), and provoke vaccination-induced virus evolution (Section 8.3.2). In human immunodeficiency virus type 1 (HIV-1), antibody-escape variants are incessantly being produced in vivo to the point that virus replication continues despite the antibody response (Richman et al., 2003; Bailey et al., 2004).

The frequency of selection of mutants that can escape a number (n) of components in which we could hypothetically separate a global immune response is far lower than the frequency of escape to a single (a, b, c, etc.) of the i components of the response. Making a simple mathematical abstraction that is applicable also to antiviral-escape mutants (Section 8.4), the frequency of mutants that escape n components of an immune response is the product of frequencies of escape to each individual component [$10^{-a} \times 10^{-b} \times 10^{-c} \times \ldots 10^{-i} = 10^{-(a+b+c+\ldots i)}$]. This is an oversimplification because it is not realistic to dissect the selective impact of a complex immune response into discrete components. A virus generally includes multiple antigenic sites and each of them is often composed of several overlapping or nonoverlapping epitopes; in addition, a virus has several T-cell epitopes in different structural and nonstructural proteins, and each epitope displays a different degree of relative dominance. However, the above abstraction reflects the advantage of stimulating the host immune system with a sufficiently broad array of B- and T-cell epitopes to prevent selection of vaccine-escape mutants due to a high genetic and phenotypic barrier (compare with the barrier to drug resistance described in Section 8.4.2). Therefore, selection of vaccine-escape viral mutants is more likely with synthetic peptidic vaccines, than with whole virus-attenuated or inactivated vaccines because the latter present a broad epitope repertoire to the immune system. Selection of escape-mutants by peptidic vaccines that evoked partial protection of cattle was documented with FMDV (Taboga et al., 1997; Tami et al., 2003). The arguments in favor of multiepitopic presentation are also endorsed by a notorious scarcity of licensed peptidic vaccines for viral diseases despite horrendous economic investments (orders of magnitude greater than investments in quasispecies research!). Use of a complex, multiepitopic vaccine, however, need not prevent long-term selection of antigenic virus variants as a result of vaccine usage, an important still largely underexplored topic discussed in Section 8.3.2.

Experimental evolution with FMDV has opened the way to a new generation of antiviral vaccines that share features of attenuated and inactivated vaccines. This new design is based on the conversion of the monopartite FMDV genome into a segmented genome version that dominated the population after extensive high MOI passages (Section 2.12 in Chapter 2 and Section 6.6 in Chapter 6). For the segmented virus version to be able to produce progeny, the two genome classes (which are encapsidated into separate particles) must reach the same cell. Therefore, administration of the segmented virus preparation should lead to an

immune response akin to that evoked by inactivated viral particles, followed by a self-limiting infection. The vaccine was tested successfully in mice and swine, the authentic host of the parental virus (Rodriguez-Calvo et al., 2010). Potential concerns with this type of vaccine are that the standard (monopartite) genome can be reconstructed by recombination in the early stages of replication in the animal. A safety level is included in that particular FMD vaccine because of multiple mutations that accumulated during the transition toward genome segmentation, which deviated the genome sequence from the one present in the original swine isolate (Moreno et al., 2014). Exploration of evolutionary mechanisms that result in altered forms of viruses may open new possibilities for vaccine design.

New prospects for attenuated vaccines have been opened with the engineering of viruses with suboptimal replication fidelity or deoptimized codon or codon pair usage (Coleman et al., 2008; Vignuzzi et al., 2008; Cheng et al., 2015). Altered polymerase copying fidelity often leads to virus attenuation (Gnadig et al., 2012; Graham et al., 2012; Korbouk et al., 2014; Rozen-Gagnon et al., 2014; Van Slyke et al., 2015). A critical issue with live-attenuated vaccines based on deviation from the standard mutation rate is the stability of the attenuation trait. Not only true revertants or other site revertants of the polymerase may arise and displace the vaccine virus, but other viral proteins may affect nucleotide incorporation (Smith et al., 2013, 2015; Stapleford et al., 2015; Agudo et al., 2016).

8.3.2 Vaccination-induced evolution

When a virus circulates in a population where vaccinated and unvaccinated host individuals coexist, and the vaccine does not induce sterilizing immunity, viruses with an altered antigenic profile might be selected. The larger the overall effective population size of the circulating virus, and the longer the virus is allowed to replicate in such a scenario, the higher the probability of incorporation of compensatory mutations that yield high-fitness antigenic variants.

These events in the case of vaccines used in veterinary medicine are particularly significant because they may alter the cell tropism and host range of viruses, thus increasing the possibilities of their zoonotic transmission into humans (Schat and Baranowski, 2007). Evidence of vaccination-induced DNA and RNA virus evolution is increasing, and it has been documented with bovine respiratory syncytial virus, bovine herpesvirus-1, Marek's disease virus, porcine circovirus 2, and classical swine fever virus, among others [(Valarcher et al., 2000; Muylkens et al., 2006; Ji et al., 2014; Kekarainen et al., 2014; Constans et al., 2015; Yoo et al., 2018); reviews in Gandon et al., 2003; Schat and Baranowski, 2007)]. The timing of dominance of CTL-escape mutants of the simian immunodeficiency virus (SIV) was influenced by vaccination, and the process could be analyzed by penetration into the mutant spectra of the relevant viral populations (Loh et al., 2008).

For human viruses, evidence of vaccine-escape mutants has been obtained for hepatitis A and B viruses. Vaccination-associated escape mutants of HAV with substitutions around the immunodominant site of the virus were identified in a cohort of HIV-1, HAV doubly infected individuals (Perez-Sautu et al., 2011). The study suggested that an incomplete vaccination schedule, combined with the HIV-1-produced immunosuppression might have contributed to high-HAV loads, thus facilitating the generation and dominance of antigenic variants. In Taiwan, the prevalence of mutants at a major antigenic determinant of the surface antigen of hepatitis B virus (HBV) tripled in 1 decade, and it has been suggested that this increase of prevalence might be due to the ample vaccination coverage in the region (Hsu et al., 1999). Other studies also suggest the circulation of HBV mutants

associated with vaccine escape and diagnosis failures (Di Lello et al., 2019).

Vaccines can rarely afford protection to all vaccinated individuals due to many factors that include variations in vaccine receptivity factors due to polymorphisms in genes involved in the adaptive immune response, immunosuppression of the vaccine recipient, the insufficient time between vaccination and exposure to the viral pathogen, and antigenic differences between the vaccine strain and circulating viruses. In addition, for massive vaccinations in veterinary medicine, damage to the vaccine (during transport, storage, etc.) and improper administration are additional problems. Vaccination may occasionally promote the selection not only of antigenic variants but also host cell tropism, host range, or virulent variants (Swayne and Kapczynski, 2008; Kirkwood, 2010; Read et al., 2015; Rolland, 2019). It is not known to what extent the widespread use of vaccination can contribute to antigenic variation relative to other factors (persistence of antibodies from previous infections, genetic drift due to genetic bottlenecks, etc.). However, our current understanding of virus dynamics should encourage investigations on the genetic and antigenic modifications of breakthrough viruses that arise from vaccinated individuals as compared with changes in viruses from unvaccinated host populations.

Reversion of live-attenuated vaccine viruses into virulent forms is a cause of disease derived from the evolutionary potential of viruses. In the case of attenuated Sabin poliovirus vaccine, the rate of vaccine-associated poliomyelitis among those vaccinated for the first time was one per 500,000 to 750,000 vaccinees, and the rate of those receiving the second vaccine dose was about one in 12 million (reviewed in Rowlands and Minor, 2010). Attenuated antiFMD vaccines were used in some countries during the second half of the 20th century, but a reversion to virulence forced the halting of the vaccination programs.

Vaccine-escape mutants may arise due to ineffective vaccines, and concomitant factors, such as immunosuppression. The escape mutants may remain confined to the unsuccessfully vaccinated host or may spread to other susceptible individuals, and attain different degrees of epidemiological relevance. Escape mutants may be direct mutants of the infecting virus or may originate by recombination between the infecting virus and other coinfecting related viruses, as observed with poliovirus and bovine herpesvirus-1 [Kew et al., 2002; Thiry et al., 2006; among other studies]. Reiteration of vaccine selection and fitness increase processes over many generations of vaccinees (be it humans or animals) may result in accelerated virus evolution. Since systematic use of vaccines for humans and animals in intensive production units is relatively recent in terms of evolutionary time (less than 100 years, and in some cases even only a few decades), it is still premature to evaluate whether vaccination is a significant factor in promoting long-term virus evolution.

8.4 Resistance to antiviral inhibitors

The first description of virus resistant to an antiviral inhibitor was by J. Barrera-Oro, H.J. Eggers, I. Tamm, and colleagues working with enteroviruses and guanidine hydrochloride and 2-(alpha-hydroxybenzyl)-benzimidazole as inhibitors (Eggers and Tamm, 1961; Melnick et al., 1961). These early results that suggested that antiviral-resistant mutants could be readily selected have been amply confirmed with many viruses and inhibitors in cell culture and in vivo. Indeed, the selection of viral mutants resistant to antiviral agents is an extremely frequent occurrence that has been known for decades, although it became widely recognized in the course of development and clinical use of antiretroviral agents to treat HIV-1 infections and AIDS.

The description of drug-escape mutants has been based on three main groups of observations:

- Detection of antiviral-resistant mutants in patients during treatment. When a reverse genetics system is available, the suspected mutation should be introduced in an infectious clone and resistance ascertained and quantified in cell culture or in vitro enzyme assays.
- Selection of resistant mutants in cell culture, by subjecting the viruses to passages in the presence of inhibitors. The viral population size is an important variable in this type of experiment (Section 8.4.1).
- Calculation of the frequency of resistant mutants by plating a virus in the absence and presence of the antiviral agent similar to the assays to calculate the frequency of monoclonal antibody (MAb)-resistant mutants (described in Chapter 7, Section 7.4.2).

In the three groups of observations, the frequency at which a specific escape mutant is found depends on a number of barriers to resistance (Section 8.4.2).

Traditionally, the fact that a drug can select virus-resistant mutants is regarded as a proof of the selectivity of the drug, as opposed to unspecific or toxic effects on the host cell that indirectly impair virus replication (Herrmann and Herrmann, 1977; Golan and Tashjian, 2011). Selection of viral mutants resistant to antiviral inhibitors is a major problem for the control of viral disease for two main reasons: (i) because it often results in virus breakthrough (increase of viral load) resulting in treatment failure and (ii) because resistant virus variants may become epidemiologically relevant, with the consequent decrease of inhibitor efficacy at the population level (Domingo and Holland, 1992; Huang et al., 2019).

Increasing numbers of antiviral agents have been developed based on the three-dimensional structure of viral proteins and their complexes with natural and synthetic ligands, in efforts that have engaged academic institutions and pharmaceutical companies. Others have been incorporated as a result of drug repositioning, that is, the discovery of antiviral activity of compounds licensed for other medical purposes, often with the help of computational tools (Ab Ghani et al., 2019). Antiviral agents may target viral or cellular proteins involved in any step of the virus life cycle. They may interact with virions and inhibit an early step of infection, such as the attachment to the host cell, penetration into the cell, or uncoating to liberate the genetic material of the virus inside the cell. Other agents interfere with the synthesis of viral nucleic acids or viral protein processing, particle assembly, or virus release from cells.

Selection of resistant mutants has been described for virtually any chemical type of antiviral agent directed to any step of the infectious cycle of DNA or RNA viruses, including important pathogens, such as herpesviruses, picornaviruses, IV, HBV, and hepatitis C virus (HCV). Several reviews and articles have covered the theoretical basis of drug resistance, and consequences for treatment management [as examples see (Domingo et al., 2001b) and previous versions in Progress in Drug Research (Richman, 1994, 1996; Ribeiro and Bonhoeffer, 2000; Domingo et al., 2001a, 2012; Menendez-Arias, 2013; Perales, 2018; Mokaya et al., 2018; Nitta et al., 2019; Pawlotsky, 2019), and the articles in the Current Opinion of Virology volume edited by L. Menendez-Arias and D. Richman (Menendez-Arias and Richman, 2014)]. Therefore, the general mechanisms that confer adaptability to viruses are very effective in finding drug-escape pathways through molecular mechanisms that are summarized in Section 8.5.

8.4.1 Replicative load and antiviral resistance

Considering the implications of quasispecies dynamics explained in previous chapters, the

following statement will be obvious to the reader: "If a single mutation is able to confer resistance to an antiviral agent, and the mutation does not cause a significant selective disadvantage to the virus (fitness decrease) in the considered environment, a drug-resistant virus mutant will be present in most, if not all, virus populations" (Domingo, 1989). If a virus replicates in such a way that a population size of 10^4 can never be achieved in a single population, it is extremely unlikely that any drug-resistance mutation (or any mutation associated with a phenotypic change) that is generated at a frequency of 10^{-4} or lower will be propagated from that viral population (Perales et al., 2011).

Selection of escape mutants depends on the replicative load, and the concentration of inhibitor attained at the sites of virus replication. Consider different cell or tissue compartments in which an antiviral inhibitor reaches different concentrations (exerts different intensity of selection) (Fig. 8.1). In each compartment, there are multiple replication complexes. A mutation conferring resistance to the inhibitor will occur at the same rate in each of them, assuming that the mutation rate is independent of the presence of the inhibitor. However, after its occurrence, the proportion of viral RNAs harboring the mutation will decrease depending on the inhibitor concentration. The time at which the effect of the inhibitor will be manifested depends on the inhibitor target. In the example of Fig. 8.1 we assume that the concentration of inhibitor-resistant mutants will decrease in the replication complexes, reaching resistant mutant frequencies of 10^{-3}, 10^{-4}, and 10^{-5} in compartments 1, 2, and 3, respectively. The frequency of inhibitor-resistant mutants in the entire cell, tissue, or organism at that time will be given by the weighted average of mutation frequencies at the individual compartments. In the case of a virus-producing viremia, assuming no bottleneck effects or differential selection for other

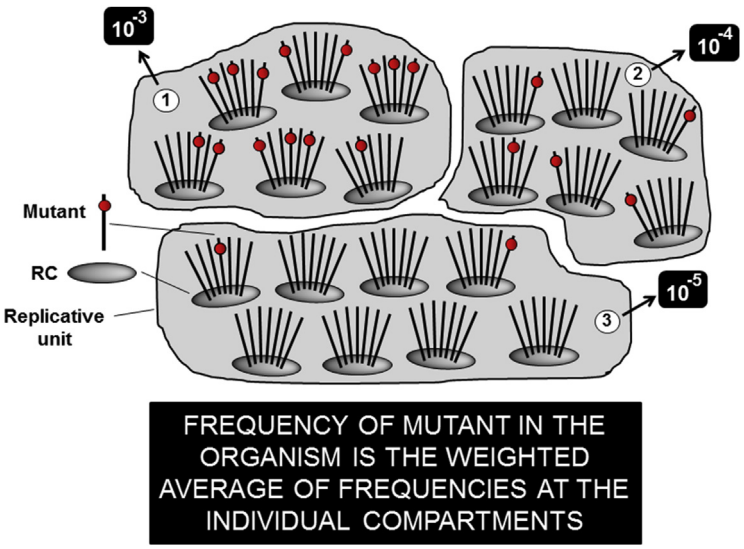

FIGURE 8.1 Frequency of a drug-resistant mutant in different compartments (subcellular site, cell, tissue, or organ) of the same organism. Three compartments labeled 1, 2, and three are drawn. Replication complexes are depicted as ellipses and replicating genomes as lines. A replicative unit is defined here as a set of replication complexes. The inhibitor-resistant mutant is represented by a red circle in a genome. Compartments 1, 2, and 3 reach increasing concentration of the inhibitor, rendering inhibitor-resistant mutant frequencies of 10^{-3}, 10^{-4}, and 10^{-5}, respectively. See text for the difference between occurrence and presence of the resistant mutant, and implications of compartmentalization.

traits, the frequency of resistant mutants calculated for the virus in blood should reflect the average frequency in all compartments that supply virus to blood. Low inhibitor concentration in a compartment will favor the selection of the resistant mutant that can either be archived as an adaptive reservoir or penetrate other compartments, depending on the sequence of events of virus spread.

If two or more independent mutations can confer resistance to an inhibitor, the probability of occurrence of an inhibitor-resistant mutant is equal to the sum of probabilities of occurrence of each mutation. For multiple mutations, the probability will be the sum of probabilities of the different mutations, a frequent case in viruses since they often display several evolutionary pathways to drug resistance. The probability of finding a viral genome resistant to two or more inhibitors directed to different targets is given by the product of probabilities of resistance to each of the individual inhibitors. The basic probability considerations regarding the frequency of occurrence of inhibitor-resistant viral mutants are summarized in Box 8.2. When two or more mutations occur in the same genome, they may be subjected to epistatic effects, meaning either increase (positive epistasis) or decrease (negative epistasis) of viral fitness (see Section 2.3 of Chapter 2 for the concept of epistasis).

The diversity of chemical structures of the antiviral compounds that can select for escape mutants is illustrated in Figs. 8.2 and 8.3 with the formulae of some antiviral agents in current or historical use. They include relatively simple organic molecules, nucleoside analogs, and complex heterocyclic compounds with a variety of residues (CH_3–, C=O, NH, NH_2, F, and Cl) that may contribute to interactions with viral proteins or alter the electronic structure of neighbor bonds thus modifying the interaction behavior of some atoms. For all of them, resistant viral mutants have been identified, despite barriers imposed upon the virus to reach a drug-resistance phenotype.

BOX 8.2

PROBABILITY OF SELECTION OF INHIBITOR-ESCAPE MUTANTS

- If there are two or more different mutations that produce the same inhibitor-resistance phenotype, and once one of the mutations is present additional mutations are no longer necessary to produce the phenotype, the probability of achieving the phenotypic change is equal to the sum of probabilities of finding each mutation individually.
- If two or more independent mutations must happen to produce resistance to an inhibitor, the probability of occurrence of the necessary mutations is equal to the product of probabilities of occurrence of each mutation individually.
- If a virus is inhibited by an inhibitor combination, and the mutations that confer resistance to each inhibitor are independent (no cross-resistance is involved), the probability of a combination-resistant mutant to arise is equal to the product of probabilities of resistance to the individual mutations.

These probability calculations are applicable to other mutation-dependent virus variations.

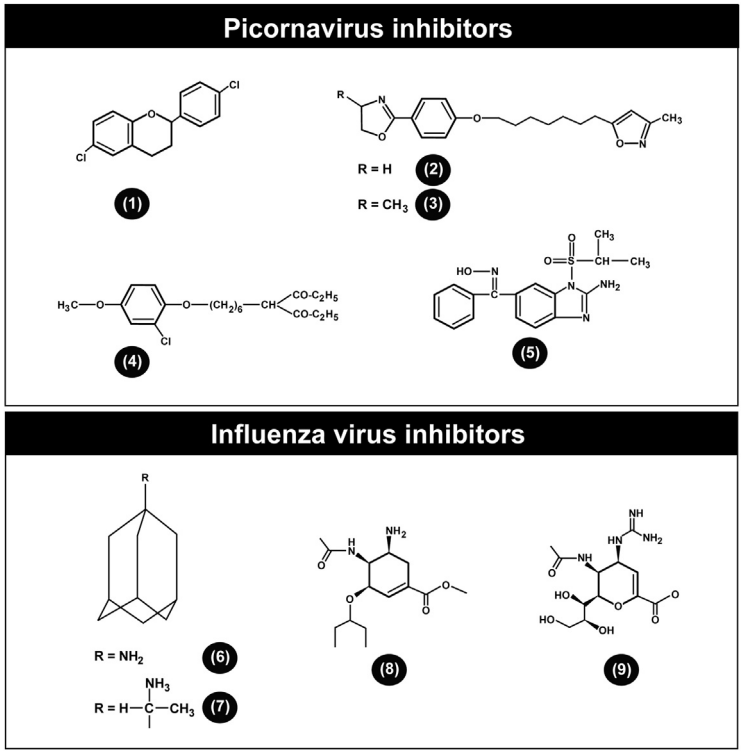

FIGURE 8.2 Some inhibitors of picornaviruses and influenza virus. The inhibitors are (1) Dichloroflavan (4′,6-dichloroflavan). (2) Disoxaril, 5-[7-[4-(4,5 dihydro-2-oxazolyl) phenoxyl] heptyl]-3-methyl-isoxazole (WIN 51,711). (3) WIN 52,084. (4) Arildone, 4-[6-(2-chloro-4-methoxyphenoxy) hexyl]-3,5-heptanedione. (5) Enviroxime, anti-6-[(hydroxyimino)-phenyl]-1-[(−methylethyl) sulfonylimidazol-2-amine]. (6) Amantadine, (1-amino-adamantane). (7) Rimantadine, (α-methyl-1-adamantane methylamine). (8) Oseltamivir (trade name Tamiflu), ethyl (3R, 4R, 5S)-5-amino-4-acetamido-3-(pentan-3-yloxy)-cyclohex-1-ene-1-carboxylate. (9) Zanamivir (trade name Relenza), (2R, 3R, 4S)-4-guanidino-3-(prop-1-en-2-ylamino)-2-((1R, 2R)-1,2,3-trihydroxypropyl)-3,4-dihydro-2*H*-pyran-6-carboxylic acid.

8.4.2 Barriers to drug resistance

The impediments for a virus to attain resistance to an inhibitor are divided into genetic, phenotypic, and mutant swarm (population) barriers to resistance (Box 8.3).

The genetic barrier to resistance to a specific inhibitor is not a universal value for a virus group, since it may be affected by genetic differences among natural viral isolates. The diversification of HCV into genotypes 1a and 1b influenced the genetic barrier to resistance to the NS3/4A protease inhibitor telaprevir [formula (20) in Fig. 8.3]. One of the amino acid substitutions that confer resistance to telaprevir is R155K in NS3. In genotype 1a, the triplet encoding R155 is AGA; therefore, a single nucleotide transition G → A can yield the triplet AAA, which encodes K. In genotype 2b, the triplet encoding R-155 is CGA; therefore, two nucleotide changes (transversion C → A and transition G → A) are required to reach AAA, the triplet encoding K. Reaching the alternative AAG codon for K would require the same or a larger number of mutations (see Section 4.3.1 in Chapter 4 for another example of how the synonymous codon usage can influence an

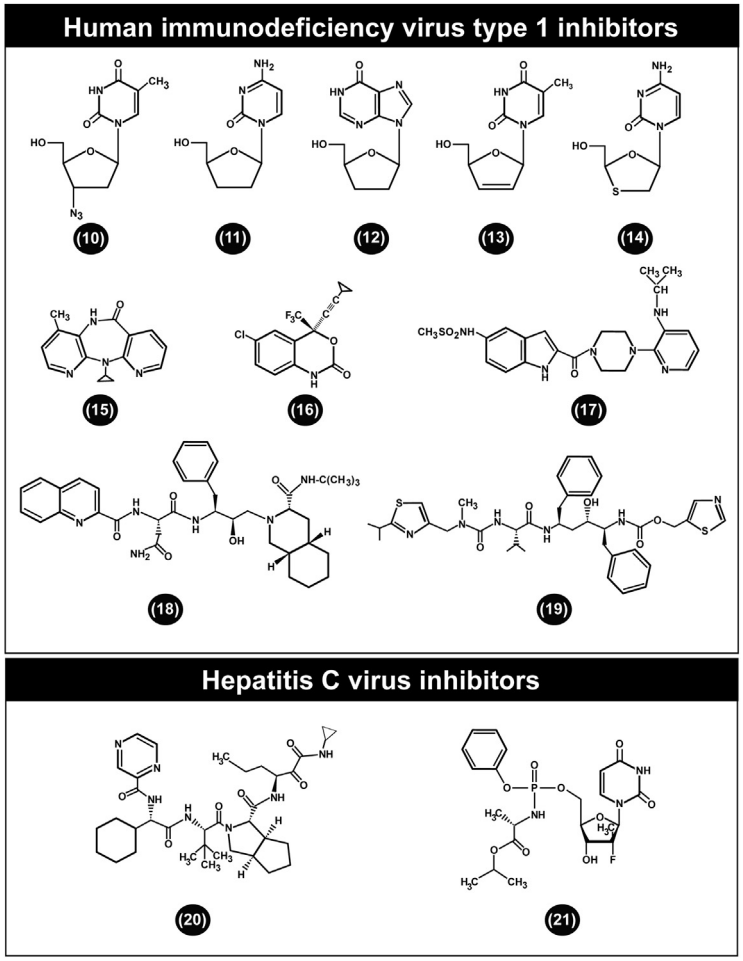

FIGURE 8.3 Some inhibitors of human immunodeficiency virus type 1 (antiretroviral agents) and hepatitis C virus. The inhibitors are (10) Zidovudine (AZT), 1-[(2R, 4S, 5S)-4-Azido-5(hydroxymethyl)oxolan-2-yl]-5-methylpyrimidine-2,4-dione. (11) Zalcitabine (ddC), 4-amino-1-((2R, 5S)-5-(hydroxymethyl)tetrahydrofuran-2-yl)pyrimidin-2(1H)-one. (12) Didanosine (ddI), 9-((2R, 5S)-5-(hydroxymethyl) tetrahydrofuran-2-yl)-3H-purin-6(9H)-one. (13) Stavudine (d4T), 1-[(2R, 5S)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-mehyl-1,2,3,4-tetrahydropyrimidine-2,4-dione. (14) Lamivudine (3 TC), 4-amino-1-[(2R, 5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one. (15) Nevirapine, 11-cyclopropyl-4-methyl-5, 11-dihydro-6H-dipyrido[3,2-*b*:2′,3′-e][1,4] diazepin-6-one. (16) Efavirenz, (4S)-6-chloro-4-(2-cyclopropylethynyl)-4-(trifluoromethyl)-2,4-dyhidro-1*H*,3,1-benzoxazin-2-one. (17) Delavirdine, N-[2-({4-[3-(propan-2-ylamino) pyridin-2-yl] piperazin-1-yl} carbonyl)-1*H*-indol-5-yl]methanesulfonamide. (18) Saquinavir, (2S)—N-[(2S,3R)-4-[(3S)-3-(*tert*-butylcarbamoyl)-decahydroisoquinolin-2-yl]-3-hydroxy-1-phenylbutan-2-yl]-2-(quinolin-2-ylformamido)butanediamide. (19) Ritonavir, 1,3-thiazol-5-ylmethyl *N*-[(2S,3S, 5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl] methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate. (20) Telaprevir, (1S,3a*R*,6aS)-2-[(2S)-2-[[(2S)-2-cyclohexyl-2-(pyrazine-2-carbonylamino) acetyl]amino]-3,3-dimethylbutanoyl]-N-[(3S)-1-1(cyclopropylamino)-1,2-dioxohexan-3-yl]-3,3a,4,5,6,6a-hexahydro-1H-cyclopenta [c]pyrrole-1-carboxamide. (21) Sofosbuvir, isopropyl(2S)-2[[[(2R,*3R*,*4R*,*5R*)-5-(2,4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyl-tetrahydrofuran-2-yl]methoxy-phenoxy-phosphoryl]amino]propanoate. Additional drugs currently used in antiviral therapy can be found in the references quoted in the text and in Chapter 9.

BOX 8.3

BARRIERS TO DRUG RESISTANCE IN VIRUSES

- Genetic barrier: Number and types of mutations needed to acquire the resistance trait.
- Phenotypic barrier: Fitness cost imposed by the resistance mutations. The cost may be due to effects at the RNA level, protein level, or both. A high fitness cost may result in reversion of the relevant mutation in the absence of the drug or incorporation of compensatory mutations that increase viral fitness.
- Mutant swarm barrier: Suppressive effect of mutant spectra may impede the dominance of resistant mutants.
- The combined effect of the different barrier classes determines the ease of dominance of drug-resistant mutants in populations subjected to the selective pressure of one or multiple inhibitors.

evolutionary outcome). The requirement of transitions versus transversions to reach the resistant phenotype will affect the genetic barrier to resistance. Most viral polymerases tend to produce transition mutations more readily than transversions presumably because in the course of RNA elongation it is easier to misincorporate a purine by another purine than by a pyrimidine, and the same for pyrimidine misincorporations (Chapter 2). Mutation preference is one of several factors that determine the frequency of drug-escape mutants. Thus, evolution may diversify viruses to display different genetic barriers to the same drugs. Since in many cases, several independent mutations may confer resistance to the same drug to complicate matters even more, it also has to be considered that the genetic barrier to one inhibitor may be affected by the presence of other inhibitors (Beerenwinkel et al., 2005).

The phenotypic barrier to drug resistance is equivalent to the fitness cost inflicted upon the virus by the mutations and corresponding amino acid substitution(s) required for resistance [Fitness cost is treated in Chapter 4 (Section 4.6) and in Chapter 7 (Section 7.4.2) in connection with the frequency of monoclonal antibody- or cytotoxic T-cell-escape mutants in viral populations]. When a drug-resistance mutation inflicts a high fitness cost, a likely result is a reversion of the mutation when the virus replicates in the absence of the drug. An alternative outcome is that compensatory mutations are introduced in the genome so that viral fitness increases while maintaining the inhibitor-resistance mutation. The two outcomes are not mutually exclusive and may contribute to the multiple, transient selection pathways observed by the application of deep sequencing to monitor the response of a viral population to specific selective force (Tsibris et al., 2009; Fischer et al., 2010; Cale et al., 2011; Kortenhoeven et al., 2015) (see Section 6.3 in Chapter 6). A high fitness cost may prevent or delay the selection of escape mutants. Sofosbuvir [formula (21) in Fig. 8.3] is a very effective NS5B (viral polymerase) inhibitor of HCV. Amino acid substitution S282T in NS5B has been associated with sofosbuvir resistance, and the substitution has been detected in patients and in some natural isolates of HCV. In one of several clinical studies on sofosbuvir efficacy, the mutant spectrum composition of HCV genotype 2b in an

infected patient treated with the drug was followed by deep sequencing of the virus at baseline (prior to initiation of treatment), in the course of treatment, and posttreatment. The frequency of S282T was 0.05% at baseline, indicating preexistence of resistance mutations despite no exposure of the virus to the drug (Section 8.6). Two days after initiation of sofosbuvir treatment, the level of S282T decreased to 0.03%, and viral breakthrough was detected 4 weeks later when 99.8% of the viral population included S282T. During the posttreatment period, genomes with the wild-type S282 amino acid regained dominance that was attributed to the true reversion of mutant genomes rather than the outgrowth of baseline wild-type genomes (Hedskog et al., 2015). This result suggests a high phenotypic barrier for sofosbuvir, and that HCV has mechanisms to overcome the barrier. The complexities of virus-host interactions render the elucidation of the pathways exploited by a virus to overcome the phenotypic barrier to a drug a highly empirical endeavor. The hope is that a combination of inhibitors that display a high barrier to resistance may impede escape and drive the virus to extinction (Chapter 9).

The mutant swarm barrier to resistance is a consequence of the interfering interactions that operate within quasispecies, and that are described in Chapter 3 (Section 3.8). It is a particular case of interference that can delay or impede the increase of frequency of a resistance mutation (Crowder and Kirkegaard, 2005; Kirkegaard et al., 2016). The possible contribution of mutant swarms to facilitate or prevent the dominance of drug-resistant mutants in infected patients is still largely unexplored.

It is difficult to anticipate how the three types of barrier listed in Box 8.3 may result in a level of drug resistance for a particular virus, in a particular host individual, in a specific target organ, at a given time. Additional influences are drug pharmacokinetics, drug penetration into different cells, tissues, and organs where the virus replicates (see Fig. 8.1), and prior history of virus replication in the infected host. It is not surprising that the study of drug resistance in viruses remains fundamentally descriptive.

8.4.3 Drug efficacy, mutant frequencies, and selection of escape mutants

When independent amino acid substitutions can lead to resistance to the same drug, alternative evolutionary pathways may be followed depending on tRNA abundances, mutational preferences, and relative nucleotide substrate concentrations at the virus replication sites. If resistance requires two or more amino acid substitutions, the genetic barrier will be correspondingly increased (Section 8.4.1 and Box 8.2).

Quantification of barriers to resistance in experiments in cell culture requires a prior characterization of the drug to be tested when acting on the cell culture-adapted virus as it infects a specific cell line. The two basic parameters to be determined are the toxicity of the drug for the host cell, and its capacity to inhibit the production of infectious virus. Toxicity is quantified by the concentration of drug that kills a given percentage (generally 50%, but sometimes another value) of cells under the conditions used in the infection. It is expressed as the cytotoxic concentration 50 (CC_{50}), as depicted in Fig. 8.4. Toxicity may depend on the cell concentration, the extent of confluence in a cell monolayer, and the metabolic state of the cell (resting vs. actively dividing). The capacity of inhibition is quantified by the concentration of inhibitor that reduces the infectious progeny production by a given percentage (generally 50%, but sometimes another value) under the defined conditions of the infection, including a multiplicity of infection (MOI). It is expressed as the inhibitory concentration 50 (IC_{50}), as depicted in Fig. 8.4. The therapeutic index (TI) is given by the quotient CC_{50}/IC_{50}, and although generally used for in vivo experiments of drug efficacy testing, it can also be applied to cell culture measurements.

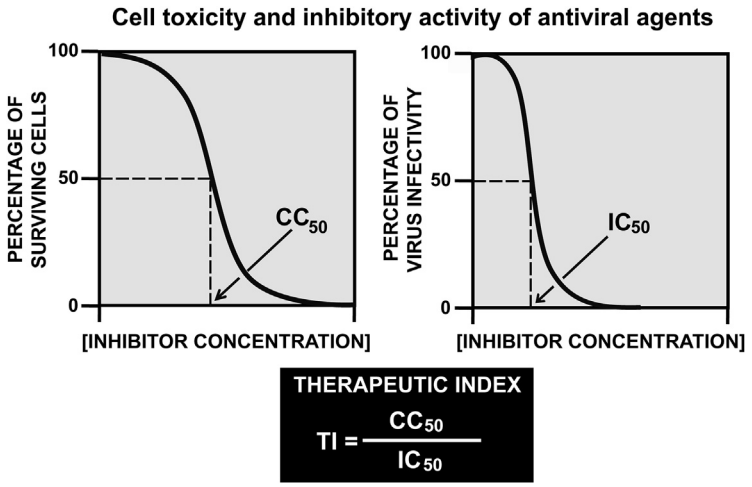

FIGURE 8.4 Schematic representation of two experiments to determine the concentration of an inhibitor needed to kill 50% of cells in culture (CC_{50} value, left) and the concentration of inhibitor that reduces the viral production to 50% (IC_{50} value, right). The therapeutic index is the quotient between CC_{50} and IC_{50} (box at the bottom). Similar tests can be performed with tissue explants or animals, under controlled environmental conditions. See text for pharmacological implications.

The three parameters, CC_{50}, $IC_{50,}$ and TI, are not universal for a virus and a drug since they may be influenced by the composition of the viral population and environmental factors, as repeatedly expressed for other features of viruses in the present book. As a guide, TI values of 100 or more suggest the excellent performance of an antiviral agent; values higher than ten are acceptable, but values lower than ten predict limited efficacy. The quantitative effects of a drug may vary when analyzing a single round of infection versus multiple rounds in serial passages, or when comparing in vivo versus cell culture experiments. CC_{50} and IC_{50} values serve as a guide to decide the range of the drug concentration to be used in serial passage experiments to evaluate the possible selection of inhibitor-resistant mutants and to estimate the genetic barrier.

The possibility to overcome a genetic barrier depends on the virus population size. For viruses that replicate in cell culture, it is possible to estimate the minimal viral population size needed to select a drug-resistant mutant which is generally positively correlated with the genetic barrier (Fig. 8.5). In the hypothetical example of the figure, a viral population is composed of inhibitor-sensitive viruses (blue spheres), and a low level of inhibitor-resistant viruses (red spheres). The proportion of inhibitor-resistant viruses is given by the mutational pressure (e.g., at a frequency of 10^{-4}, which is increased in the picture for clarity). Passage of a small amount of virus (e.g., 10^2 infectious virus in the small circle at the upper part of the figure) will exclude the mutant virus (red spheres) that will be maintained at the basal level dictated by mutational pressure in the course of passages (limited to two in the figure for simplicity). Selection of escape mutants is precluded by the limited population size at each transfer. In contrast, if the population size used for the successive infections is sufficiently large ($>10^4$, larger circles at the bottom that surround both sensitive and resistant viruses), the resistant mutant can become

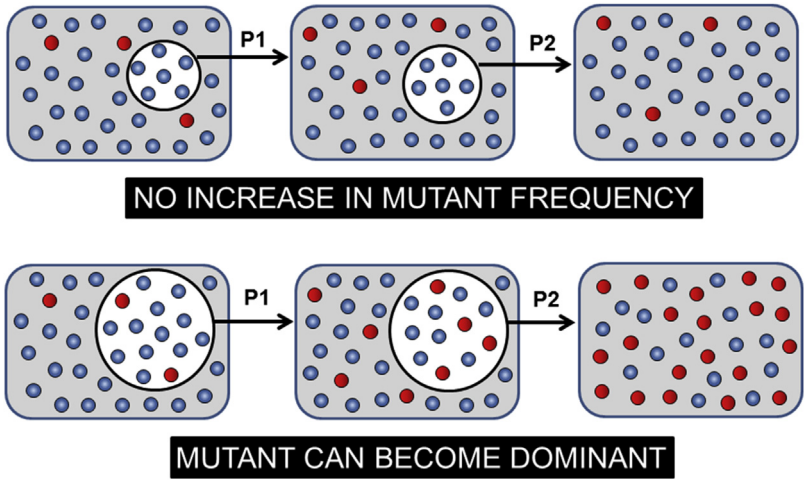

FIGURE 8.5 The effect of sampled virus population size on the dominance of an inhibitor-resistant mutant. The upper left population is composed of inhibitor-sensitive (blue spheres) and inhibitor-resistant (red spheres) viruses. If during passages P1 and P2, the amount of sampled virus is insufficient (upper three successive populations), the resistant virus mutant will not be enriched in the population. If during passages P1 and P2, the amount of sampled virus exceeds a critical value (bottom three successive populations), the population will be enriched in the resistant mutant. See text for numerical examples.

gradually dominant and can be isolated for further studies. To give another example, a single amino acid replacement that requires two mutations (the change from CAG to AUG to attain substitution Q151M in HIV-1 reverse transcriptase, associated with resistance to multiple antiretroviral nucleosides) will occur at a lower frequency than replacements that require a single mutation. If each of the two mutations reaches a frequency of 2×10^{-4}, the expected frequency of the drug-resistant genomes (ignoring fitness effects) will be $(2 \times 10^{-4}) \times (2 \times 10^{-4}) = 4 \times 10^{-8}$. Thus, at least 4×10^{8} viral genomes must undergo one round of copying (or a lower number of genomes a proportionally higher number of rounds of copying) to approach a good probability to obtain a drug-resistant genome in that viral population. Population size limitation of a drug selection event is a specific example of how random events may intervene in the process of positive selection (compare with Section 3.2 and Fig. 3.2 in Chapter 3; in that figure, the random event that excludes the positively selected population is conceptually equivalent to the insufficient population size depicted in the upper infection series of Fig. 8.5).

When two or more mutations are needed to confer the resistance phenotype, drug resistance will be less likely not only due to the lower probability of generating the two required mutations but also because of the increased chances of two mutations in the same genome entailing a fitness cost. A virus that requires three or more mutations to overcome a selective constraint may occur at a frequency in the range of 10^{-12} or lower which will often be insufficient for the mutant to be present in the mutant spectrum of the infected host (Fig. 8.6).

Failure to select for a drug-resistant mutant in cell culture does not necessarily mean that the resistant mutant is not present in the population. It may mean that due to a high genetic barrier, the selection experiment was designed to infect with an insufficient amount of virus. Similar and even more accentuated problems are encountered in selection experiments in vivo, since not only

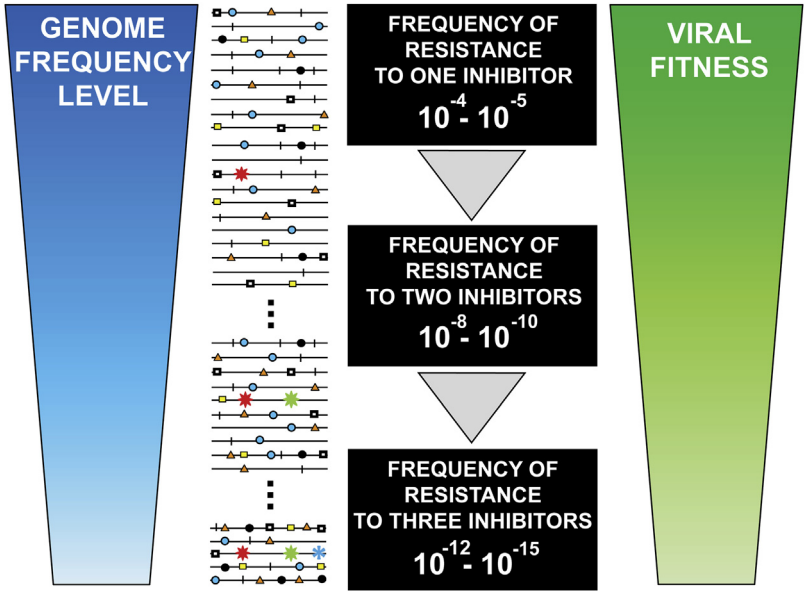

FIGURE 8.6 Decreased frequency and fitness of mutant genomes resistant to one, two, or three inhibitors. The genome frequency level decreases by several orders of magnitude when resistance to one inhibitor (red asterisk in the upper mutant spectrum), or two inhibitors (red and green asterisks in the middle-mutant spectrum), or three inhibitors (red, green, and blue asterisk in the bottom mutant spectrum) must occur in the same genome (left part of the figure and numerical values at the center). Increased number of mutations generally implies fitness decrease (right part of the figure). See text for implications.

population size but also the source of the virus (blood or another organ) for the next infection may determine the inclusion or exclusion of the relevant mutation (compare Figs. 8.1 and 8.6).

8.4.4 Phenotypic barrier and selective strength

The phenotypic barrier or fitness cost inflicted by a drug-resistance mutation (Box 8.3) is often estimated empirically from the frequency of the relevant substitution in patients treated with the drug or in cell culture assays. An adequate procedure to quantify the phenotypic barrier to resistance is to determine the fitness of the virus expressing the protein with the wild-type amino acid (the one that confers drug sensitivity) relative to the virus expressing the protein with the substituted amino acid that confers resistance; fitness is measured in the absence and presence of the drug (double assay). This is an extension of the determination of fitness vectors described in Section 5.1.1 of Chapter 5, as depicted in Fig. 8.7; the assays are best performed in cell culture, although the use of explants or in vivo assays is also feasible. Two parameters can be calculated: the fitness cost inflicted by the amino acid substitution associated with resistance, in the absence of the drug (reflected in a fitness value $f_{-\,\mathrm{DRUG}} \leq 1$ relative to the wild type), and the selective advantage conferred by the substitution in the presence of the drug (reflected in a fitness value $f_{+\,\mathrm{DRUG}} > 1$ relative to the wild type). The lower the value of $f_{-\,\mathrm{DRUG}}$, the higher the fitness cost. We define the selective strength of the resistance mutation as $f_{+\,\mathrm{DRUG}}/f_{-\,\mathrm{DRUG}}$. For example, if we put arbitrary numbers (unrelated to values shown in ordinate) to the fitness values in the first graph of Fig. 8.7,

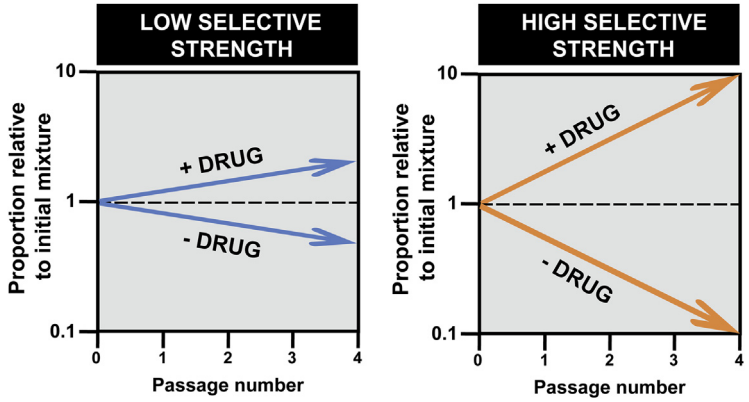

FIGURE 8.7 Selective strength of an inhibitor-resistance mutant. A mutation may confer a different degree of resistance at a different fitness cost for the virus. The fitness vectors in the left panel denote limited resistance at a low fitness cost, whereas the vectors in the right panel denote high resistance despite a considerable fitness cost inflicted by the resistance mutation. The selective strength is defined as the ratio of fitness in the presence and absence of the drug, as explained in the text.

$f_{+\,DRUG} = 1.4$ and $f_{-\,DRUG} = 0.8$, we obtain a selective strength of 1.7. For the vectors in the second graph, if $f_{+\,DRUG} = 3.6$ and $f_{-\,DRUG} = 0.1$, the selective strength is 36. High selective strength means an important selective advantage conferred by the amino acid substitution for virus replication in the presence of the drug despite a high fitness cost inflicted by the substitution (compare with Section 4.2 in Chapter 4 for the trade-off and "no free lunch" concepts). If the substitution does not entail any fitness cost ($f_{-\,DRUG} = 1$), the fitness value in the presence of the drug equals the selective strength. Selective strength can be calculated for a mutation or group of mutations that confer resistance to a drug used at a given concentration in a defined environment [example in (de la Higuera et al., 2017)]. The limitations of fitness measurements (environment dependence, etc.) described in Section 5.1.2 of Chapter 5 apply here. Since viral genomic sequences may vary in the course of fitness assays, a limited number of passages and triplicate parallel assays are recommended. If a substitution entails a high fitness cost, direct reversion of the substitution or incorporation of compensatory mutations may occur. Nucleotide sequence monitoring in the course of the assay should reinforce the conclusions.

8.4.5 Multiple pathways and evolutionary history in the acquisition of drug resistance

Most drug-resistance mutations inflict a fitness cost upon the virus and yet very rarely drug resistance represents an unsurmountable barrier to maintain viral infectivity. Several possibilities can account for the pertinacious occurrence and selection of drug-resistant, viable viral mutants. One possibility, supported by some experimental and clinical observations, is that a drug-resistant phenotype may be achieved through a number of alternative genetic modifications. Even if a specific amino acid substitution—that would serve as the most direct and effective determinant of drug resistance—were highly detrimental or lethal for the virus, alternative mutations can often be found that lead to a similar resistance phenotype, or at least a sufficient resistance to permit virus replication and exploration of sequence space to find compensatory mutations.

The connectivity among points of sequence space and the fact that several space positions map into the same (or similar) drug-resistance phenotype contribute to the extended occurrence of drug resistance. This phenotypic redundancy applies to both standard nonmutagenic inhibitors and to mutagenic inhibitors. The cascade of mutations that confer resistance of picornaviruses to the mutagenic purine analog ribavirin illustrates how alternative amino acid substitutions in the viral polymerase (some being genuine resistance mutations and others acting as compensatory substitutions to maintain polymerase function) can lead to the ribavirin-resistance phenotype (discussed in Chapter 9).

A speculative interpretation of the systematic occurrence of drug-resistant viral mutants is that the majority of the chemicals used in antiviral therapy (Figs. 8.2 and 8.3) have a structure which may be related to natural compounds that viruses and their ancestral replicative machineries encountered in their continuous struggle to survive. In this view, drug resistance would have been gradually built as a consequence of coevolution (Section 4.5 of Chapter 4) between virus replicative and gene expression machineries and the "space" of chemical compounds that interacted with them. Mechanisms of drug resistance might have had their roots in molecular events repeatedly experienced as viruses evolved in an interactive manner with protocellular and cellular metabolites in our biosphere. Discrimination in favor of small molecule substrates compatible with a flow of genome replication and gene expression and avoidance of perturbing intruders that could alter catalytic activities should have been positively selected. Unfortunately, this is a possibility we will never be able to test. Whatever the reasons behind, the unfortunate reality is that drug resistance is an extremely frequent event that complicates enormously the control of the viral disease.

8.5 Molecular mechanisms of antiviral resistance

Multiple drug evasion mechanisms have been identified or proposed for RNA and DNA viruses, and they can operate depending on basic replicative parameters in connection with drug levels and their variation with time. Delayed lysis of bacteriophage ϕX174 that reduced the replication rounds in the presence of 5-fluorouracil (FU) produced resistance to this analog (Pereira-Gómez and Sanjuán, 2014). In this line, synchronization of a virus life cycle so that replication occurs when drug levels are minimal has been proposed as a potential resistance mechanism (Neagu et al., 2018). Virus plasticity favors modification of life cycle parameters for virus survival in the presence of drugs, provided time for the relevant selection events is allowed once the treatment has been implemented. The great majority of inhibitor-resistance mechanisms involve amino acid substitutions in viral proteins that directly or indirectly diminish the binding of the drug to the viral target.

The following major mechanisms have been documented:

- Substitutions in the protein targeted by the drug that decreases the affinity of the protein for the drug. Mutations that modify nucleotide selectivity in viral polymerases belong to this group. Substitutions may affect the neighborhood of the polymerase catalytic site, other polymerase domains, or even nonstructural proteins that interact with the polymerase.
- If the inhibitor acts on a viral protein that itself has some other viral protein or genomic structure as a target, amino acid substitutions, or mutations that affect that target may also contribute to resistance. Correlated mutations

in the first and second target may also yield the resistance phenotype. This is the case of some protease inhibitor-resistance mutations in HIV-1.

- In the case of viral polymerases, some mutations permit the excision of a chain-terminating nucleotide at the 3′-end of the primer. It is achieved through phosphorolysis mediated by a pyrophosphate donor, probably ATP.

8.5.1 Some examples with HIV-1

Resistance to nucleoside/nucleotide reverse transcriptase (RT) inhibitors (NRTIs) is achieved by one of at least two mechanisms: (i) discrimination against the incorporation of the triphosphate form of the NRTI and (ii) excision of the chain-terminating nucleotide once incorporated at the 3′-end of the growing DNA chain. This occurs with thymidine analog resistance mutation (or TAMs) that are typically selected under treatment with AZT or d4T [formulae (10) and (13) in Fig. 8.3]. Groups of amino acid substitutions may yield a multidrug-resistance phenotype. A well-studied example in HIV-1 is the Q151M complex in the reverse transcriptase, which includes substitutions A62V, V75I, F77L, F116Y, and Q151M. The phenotype consists of the limitation of incorporation of several nucleotide analogs. These and other mutants are characterized by a decrease in the catalytic rate constant (k_{pol}) of incorporation of the analog or analogs relative to standard nucleotides (see Section 2.6 in Chapter 2 for the basic kinetic parameters for polymerase activity). The combination of enzymological and structural studies has provided a molecular interpretation of the mechanism of inhibition of HIV-1 by NRTIs (reviews in Menéndez-Arias, 2013; Menéndez-Arias and Alvarez, 2014; Clutter et al., 2016; Günthard et al., 2019); for a predictive model that includes nucleotide levels, see von Kleist et al. (2012).

Nonnucleoside RT inhibitors (NNRTIs) bind to an RT pocket 10Å away (a considerable distance) from the catalytic site composed of residues of the p66 and p51 subunits of the enzyme. The hydrophobic nature of NNRTIs is illustrated in Fig. 8.3 with the structures of nevirapine, efavirenz, and delavirdine [formulae (15), (16), and (17), respectively]. Several mechanisms have been proposed to explain their inhibitory activity, including alteration of the catalytic amino acids YMDD at the RT active site, distortion of the nucleotide-binding site, or modification of the position of the primer that receives the incoming nucleotides (Menéndez-Arias, 2013). Amino acid substitutions that confer resistance to NNRTIs block the access of the inhibitors to their binding sites or alter the conformation and volume of the binding pocket.

The essential viral proteases are a target for the development of specific antiviral inhibitors. Examples are saquinavir and ritonavir for the HIV-1 [formulae (18) and (19), respectively, in Fig. 8.3]. Multiple resistance mutations have been described for protease inhibitors. They can affect the substrate-binding site or neighbor positions, often accompanied of compensatory substitutions at distant positions including sites of the target viral protein [e.g., the Gag cleavage sites in HIV-1 (Fun et al., 2012; Flynn et al., 2015)]. Some HIV-1 protease inhibitor combinations display a high genetic barrier to resistance but, significantly for the capacity of viruses to explore sequence space, resistant mutants with 20−30 amino acid substitutions in the protease-coding region have been isolated (Rhee et al., 2010).

The reviews by Menéndez-Arias (2013) and Menéndez-Arias and Alvarez (2014) provide excellent and detailed accounts of mechanisms of resistance of HIV-1 and HIV-2 to inhibitors that include virus entry and integrase inhibitors, in addition to the RT and protease inhibitor briefly described here.

8.5.2 Mutation site and functional barrier

Because of the distance effects that can be exerted among amino acids on the structure of proteins, substitutions that confer resistance to inhibitors of viral enzymes may lie far from the catalytic site. The effect of drug-resistance mutations on the general catalytic efficiency of a viral enzyme is one of the determinants of the functional barrier to resistance, since it may diminish replicative fitness. When an enzyme activity assay in vitro is available, the effects of specific drug-resistance amino acid substitutions on enzyme activity can be tested, although the observed alteration may not be the only influence on the fitness modification of the corresponding mutant virus. The reason is that most viral proteins (including viral enzymes) are multifunctional, and an enzyme activity assay may not capture the range of influences exerted by the enzyme. Regarding direct-acting antiviral (DAA) agents for HCV, the inhibitors that target the HCV polymerase (NS5B) generally display a higher functional barrier to resistance than the protease inhibitors and manifest a broader genotype coverage. Fitness decreases entail reductions in viral load and, consequently, lower probability of viral breakthrough (treatment failure). Nucleotide analogs that bind to conserved residues at or near the active site of the viral polymerase tend to show subtype-independent antiviral activity. Because amino acid substitutions at or near the active site of viral enzymes are likely to inflict a fitness cost, such substitutions may not preexist in treatment-naive patients (Margeridon-Thermet and Shafer, 2010; Sarrazin and Zeuzem, 2010). Interestingly, in the case of HIV-1, mutations conferring resistance to RT inhibitors inflict a lower fitness cost than mutations that confer resistance to protease inhibitors (reviewed in Martinez-Picado and Martinez, 2008). Thus, enzymes that perform similar functions for different viruses may have evolved to display different tolerance to amino acid substitutions. It is not possible to generalize which types of resistance mutations will display high or low functional barriers.

8.5.3 Additional considerations on escape mutant frequencies

There is a broad range of frequencies of antibody- and drug-escape mutants in viral populations, although values of 10^{-4} to 10^{-6} mutants per infectious unit are frequent for many RNA and DNA viruses (see Table 7.2 in Chapter 7 for antibody-escape mutants). A few estimates for drug-escape mutants are listed in Table 8.1; several observations and characterization of escape mutants have not been accompanied by frequency measurements. A point worth emphasizing is that quantification of viruses harboring biologically relevant mutations has been possible because an adequate biological assay is available. An antiviral agent or a neutralizing antibody measures the proportion of infectious viral particles that differ from the majority of the population in the relevant resistance trait. There is no reason to suspect that the viral amino acid residues (that are the target of an inhibitor or an antibody) that are substituted to confer the resistance phenotype are more prone to accept variations than many other amino acids in viral proteins. If we had additional selective agents to probe other viral sites, we expect a similar range of variant amino acids than using inhibitors or antibodies. This quite straightforward prediction is another way to state that there is general agreement in the mutation rates and frequencies for viruses being in the range of 10^{-3} to 10^{-5} substitutions per nucleotide (s/nt), calculated using a variety of biochemical and genetic methods (Chapter 2).

A quite general observation is that in antibody neutralization experiments, a fraction of the virus population remains infectious despite the

TABLE 8.1 The frequency of some drug-escape mutants.

Drug[a]	Virus[b]	Frequency	Comments	References
HBB[c]	CAV9	10^{-4}	Reversion from HBB dependence to HBB independence	Eggers and Tamm (1965)
Amantadine and rimantadine	IV	1×10^{-3} to 4×10^{-4}	Measurements in cell culture	Appleyard (1977), Lubeck et al. (1978)
Rimantadine[a]	IV	27%	Percentage of children treated with rimantadine that shed resistant IV	Belshe et al. (1988)
Disoxaril[a]	HRV14	1×10^{-3} to 4×10^{-4}	Low-level resistance in cell culture	Heinz et al. (1989)
		4×10^{-5}	High-level resistance in cell culture	
Guanidine[d]	PV	1.8×10^{-5} to 4×10^{-8}	Measurements in cell culture	Pincus and Wimmer (1986)

[a] *The formula of these drugs is included in Figure 8.2 with the following number in parenthesis: amantadine (6); rimantadine (7); disoxaril (2).*
[b] *The virus abbreviations are CAV9, coxsackievirus type A9; IV, influenza virus; HRV14, human rhinovirus type 14; PV, poliovirus.*
[c] *HBB is 2-(alpha-hydroxybenzyl)-benzimidazole.*
[d] *Guanidine is aminoformamidine.*

addition of high antibody concentrations. Although the resistance mechanism is unclear, a possibility is that the heterogeneous viral population includes a small proportion of antigenic variants with decreased affinity for antibodies. Incomplete neutralization with the nonsigmoidal slope in the neutralization curves has been characterized for broadly neutralizing antibodies directed to HIV-1 (McCoy et al., 2015). This general observation is an added complication to preventive designs that consider administration of neutralizing antibodies either as vaccine additives or in combination with antiviral inhibitors (Section 9.2 in Chapter 9).

The calculated frequencies of antibody- or drug-resistant mutants in viral populations may be lower than the rate at which they originate by mutation due to the fitness cost of the mutation (Section 8.4.4). The argument is parallel to that used to justify why mutation rates and frequencies differ due to the fitness effects of mutations (Chapter 2). Another prediction derived from the above considerations is that mutations conferring resistance to antiviral agents are expected to be detected in viral populations never exposed to the relevant drugs. All forms of genetic variation of viruses can contribute to dominance and spread of drug-resistant mutants, including the combined effect of mutation, recombination, and genome segment reassortment (Richman, 1996; Neher and Leitner, 2010; Rogers et al., 2015). The basal level of mutational pressure may be sufficient to provide a detectable proportion of escape mutants without the need for selection by the selective agent. This is an important aspect of antiviral therapy that is addressed next.

8.6 Antiviral resistance without prior exposure to antiviral agents

The first demonstration that the baseline mutation level in viral quasispecies can include a detectable level of mutations that confer resistance to inhibitors in the absence of selection by the inhibitors, was obtained by D.D. Ho, I. Nájera, C. López-Galíndez, and their colleagues working with HIV-1 (Mohri et al., 1993; Nájera et al., 1994, 1995). One of the studies examined the *pol* gene of 60 HIV-1 genomes obtained directly from lymphocytes of infected patients. Mutation frequencies for independent viral isolates were in

the range of $1.6.10^{-2}$ to $3.4.10^{-2}$ s/nt, while for mutant spectrum components of individual isolates the values were $3.6.10^{-3}$ to $1.1.10^{-2}$ s/nt. In the virus from these patients, mutation frequencies at the codons for amino acids involved in antiretroviral resistance were very similar to the average mutation frequency for the entire *pol* gene. Consistently with the mutation frequency values, several mutations that led to amino acid substitutions that conferred resistance to reverse transcriptase inhibitors were identified in patients not subjected to therapy. At the time of the study, the number of antiretroviral agents was still limited, and a considerable number of patients were not treated. The authors gave convincing epidemiological arguments that the background of mutations related to antiretroviral resistance was a consequence of high mutation rates and quasispecies dynamics, and not due to the transmission of resistant virus from individuals that had been subjected to therapy (primary resistance) (Nájera et al., 1995).

The presence of inhibitor-resistance mutations in viral populations never exposed to the corresponding inhibitor has been confirmed for HIV-1 and for several other viruses, including HCV, and it is supported by the calculated mutant frequencies in viral quasispecies (Havlir et al., 1996; Lech et al., 1996; Ribeiro et al., 1998; Ribeiro and Bonhoeffer, 2000; Cubero et al., 2008; Johnson et al., 2008; Toni et al., 2009; Tsibris et al., 2009; Peres-da-Silva et al., 2017). Ample support has also come from deep sequencing analyses of mutant spectra, opening a point of debate on the basal frequency of inhibitor-resistance mutations that constitutes an indication to avoid the use of the corresponding inhibitors in therapy. At least in the case of HCV (and probably applicable to other viruses), there is no basis to suggest that the presence of a drug resistance mutation below a certain level in a patient will not have relevance for treatment failure when the inhibitor is administered. The understanding of quasispecies dynamics cautions against such reductionist arguments as evidenced by clinical cases (Perales et al., 2018). The data underline the relevance of mutant spectra as phenotypic reservoirs to confront selective constraints before constraints are in operation. For treatments, including a drug that has already been administered to a patient in the past, the influence of quasispecies memory should also be considered (Section 5.5.1 in Chapter 5). Mutant spectra can be viewed as an anticipatory reservoir of phenotypes.

In addition to the presence of drug resistance mutations in viral populations due to mutant frequency levels, the transmission of drug-resistant mutants from treated to naïve patients may contribute to epidemiological relevance of resistance mutations. Such primary resistance has been amply documented with HIV-1, and it appears to increase in the case of HCV (Franco et al., 2014; Echeverría et al., 2016; Huang et al., 2019). Higher levels of resistance mutations as a function of time in untreated patients is an indication that the mutations are not due to basal mutant frequencies but to the epidemiological expansion of virus mutants that originated in treated patients. From the clinical data available, the rate of expansion of virus harboring resistance mutations may vary depending on transmission and epidemiological features of each pathogen, but it seems unavoidable in the face of extended treatments for genetically variable pathogenic viruses. We confront a situation with parallels with antibiotic resistance in bacteria (Chapter 10).

8.7 Fitness or a fitness-associated trait as a multidrug-resistance mechanism

The major mechanism of drug resistance in viruses is based on amino acid substitutions that render the drug ineffective through the several molecular mechanisms summarized in Section 8.5. Application of the cell culture system of HCV replication in human hepatoma cells (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) to examine the effects of

long-term evolution has indicated that viral fitness can be an additional mechanism of drug resistance. The evidence was obtained when addressing the important issue of HCV resistance to interferon-alpha (IFN-α). IFN-α and ribavirin were the two components of the standard of care treatment against HCV infections until the advent of new therapies based on DAA agents in 2014. Natural HCV isolates differ in IFN-α sensitivity, and the molecular basis of the difference is largely unknown. The study in cell culture consisted in subjecting a clonal population of HCV (termed HCVp0, prepared by electroporation of hepatoma cells with RNA encoding the viral genome, transcribed from a plasmid) to 100 serial passages (of the type described in Section 6.1 of Chapter 6) in the absence or presence of increasing concentrations of IFN-α added to the culture medium. Several mutations scattered throughout the HCV genome were associated with IFN-α resistance (Perales et al., 2013). The selection of multiple alternative mutations is most likely influenced by the fact that IFN-α evokes a multicomponent antiviral response, which is not focused toward a single viral protein (Perales et al., 2014). Unexpectedly, even the control HCV populations (those passaged 100 times in the absence of IFN-α) displayed a partial (but statistically significant) resistance to IFN-α that could not be attributed to endogenous IFN production by the hepatoma cells (Perales et al., 2013).

In view of this intriguing result, the initial HCVp0 population and the HCV population passaged 45 and 100 times in the absence of IFN-α (termed HCVp45 and HCVp100, respectively) were tested for their resistance to other inhibitors of HCV replication: the protease inhibitor telaprevir [formula (20) in Fig. 8.3], the NS5A inhibitor daclatasvir, the cellular protein cyclophilin A inhibitor cyclosporin A, the mutagenic purine nucleoside ribavirin, and the high barrier inhibitor sofosbuvir [formula (21) in Fig. 8.3]. HCVp45 and HCVp100 displayed significantly increased resistance to all inhibitors tested, as compared with the parental population HCVp0 (Sheldon et al., 2014; Gallego et al., 2016) (Fig. 8.8). Passage of HCV entailed a 2- to 3-fold increase of viral fitness and a broadening of the mutant spectrum that might have increased the frequency of mutations associated with drug resistance, thus explaining the behavior of the multiply passaged HCV populations. The search for the resistant mutations was easier for telaprevir, daclatasvir, and cyclosporin A than for the other drugs because amino acid substitutions in the target protein had been previously identified as responsible for drug resistance. (In the case of cyclosporin A resistance, substitutions map in NS5A and NS5B, because the drug binds to cyclophilin A, which in turn interacts with NS5A). Analysis of the mutant spectra of HCVp45 and HCVp100 by molecular cloning and Sanger sequencing and by deep sequencing failed to identify specific drug-resistance mutations. Since it could not be excluded that the broadening of the mutant spectrum might have increased the frequency of resistance mutations still to be characterized, two additional tests were performed. One was to determine the kinetics of viral production over a 1000-fold range of MOI in the absence and presence of telaprevir. Both the unpassaged and multiply passaged HCV displayed parallel kinetics at the different MOIs, which excludes that drug resistance was due to the presence of resistance mutations in minority components of the mutant spectrum (Fig. 8.8). To further substantiate the findings, biological clones obtained by end-point dilution of the corresponding HCVp0 and HCVp100 populations were tested regarding drug resistance. A biological clone should have eliminated the minority genomes that harbored drug-resistance mutations since biological cloning is the most severe form of bottleneck event (Sections 6.2 and 6.5 in Chapter 6). The biological clones did not display any decrease in drug resistance as compared

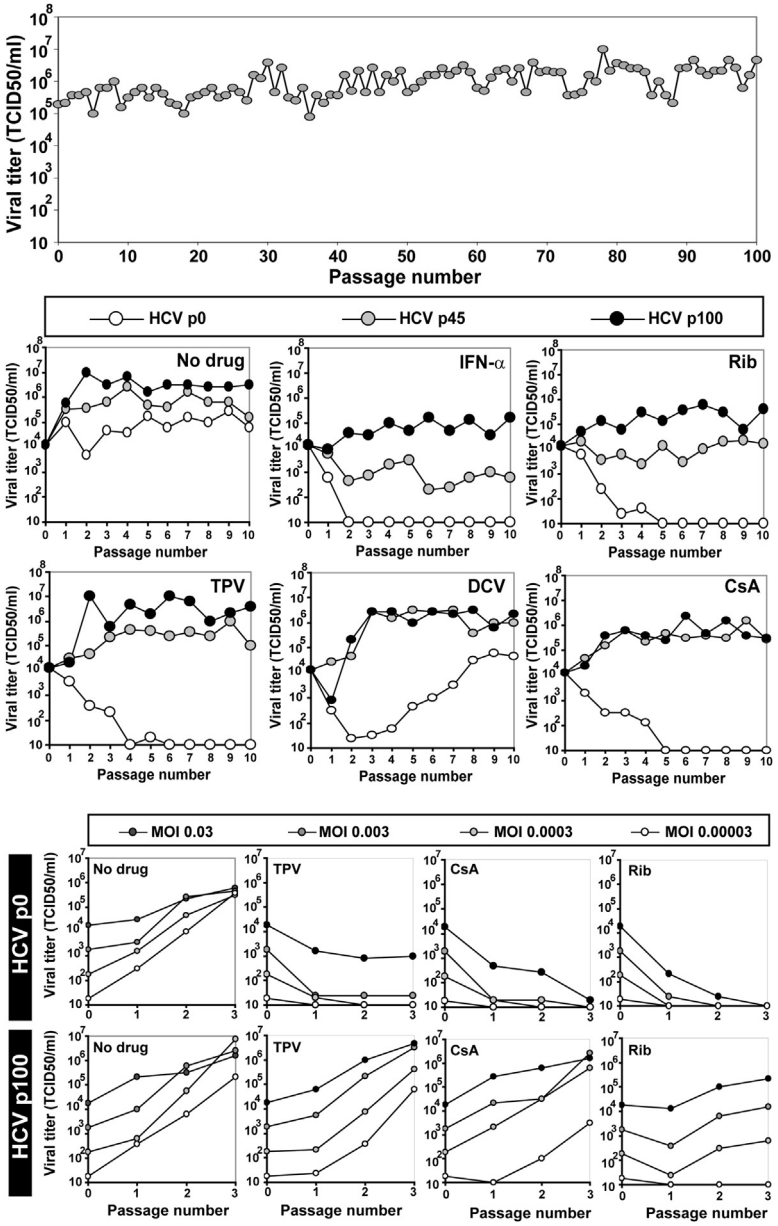

FIGURE 8.8 Multidrug resistance of hepatitis C virus passaged in human hepatoma cells. Top: Evolution of virus infectivity in the course of 100 serial passages. Middle: HCVp0, HCVp45, and HCVp100 are the initial hepatitis C virus, the HCV passaged 45 times and the virus passaged 100 times in the absence of any drug (samples from the experiment described at the top). The individual panels give the virus titer in the course of 10 passages either in the absence of any drug (No drug) or the presence of IFN-α, ribavirin (Rib), telaprevir (TPV), daclatasvir (DCV), or cyclosporin A (CsA). Note the resistance of HCVp45 and HCVp100 as compared to HCVp0 to several inhibitors. Bottom: Virus titer in the course of three serial passages of HCVp0 and HCVp100 (written in the filled boxes on the left) at the indicated multiplicity of infection (box). See text for the interpretation of these experiments and Sheldon et al. (2014) for details. *The figure has been modified from Sheldon, J., Beach, N.M., Moreno, E., Gallego, I., Pineiro, D., et al., 2014. Increased replicative fitness can lead to decreased drug sensitivity of hepatitis C virus. J. Virol. 88, 12098–12111, with permission from the American Society for Microbiology, Washington DC, USA.*

with their corresponding parental, uncloned populations (Sheldon et al., 2014).

The above observations have established viral fitness as a multidrug-resistance determinant in HCV that may also apply to other viruses. One possible molecular mechanism may consist of competition between replicative complexes and inhibitory molecules inside the infected cells. This model implies that fitness increase is reflected either in more replicating molecules per each replicative unit or in an increase in the number of replicative units per cell, without any influence on the number of inhibitor molecules that reach the replication sites. Exploration of this competition model and alternative models and the extension to other viral-host systems are important challenges in the field of antiviral research.

To sum up, mutant spectra and quasispecies dynamics can mediate antiviral resistance by at least two mechanisms: (i) by increases in the proportion of resistance mutations in the mutant spectra and (ii) by a fitness increase promoted by continued viral replication in the same environment. Both mechanisms may act conjointly during viral infections in vivo. Some studies with HCV have documented drug-resistance phenotypes in infected patients, in the absence of specific drug-resistance mutations (Sullivan et al., 2013; Svarovskaia et al., 2014; Sato et al., 2015; Stross et al., 2016; Di Maio et al., 2017; Dietz et al., 2018). In fact, prolonged chronic HCV infections represent an adequate scenario for fitness increase due to extended rounds of infections in the same host liver. As a consequence, chronic infections may be prone to display fitness-associated multidrug-resistance phenotypes in the absence of drug-resistance mutations. The multiple mechanisms of drug resistance related to quasispecies dynamics justify even further the need for new antiviral strategies, as presented in Chapter 9.

8.8 Viral load, fitness, and disease progression

High viral loads are predictors of disease progression. For HIV-1 and other lentiviruses, efficient early control of virus replication by the host immune response is generally associated with limited disease severity. The viral load that follows after the initial immune response to HIV-1 is referred to as the "viral set point." In the absence of early therapy, low set points in HIV-1 are generally attributed to a strong cellular immune response, likely influenced by additional host and viral factors. [This and other aspects of HIV-1 replication and pathogenesis have been reviewed in excellent monographs by Levy (2007, 2015)]. A low set point predicts an asymptomatic outcome, and this is generally the case for viruses that establish persistent rather than acute infections. High viral fitness during the early stages of viral replication can promote disease manifestations. This was suggested by the progression toward the disease of a cohort of individuals that were infected during blood transfusion with an HIV-1 containing a large deletion in Nef, an adaptor protein that mediates replication and pathogenesis (reviewed in Arien and Verhasselt, 2008). After more than 15 years, some of the infected individuals showed clinical signs, probably as a result of the accumulation of mutations in the HIV-1 genome that compensated for the lack of Nef. More generally, fitness-decreasing (but not lethal) genetic lesions in a viral genome may be compensated by additional genomic mutations that become increasingly dominant in the course of further viral replication. The kinetics of fitness gain will depend on the nature of the lesion and the functional implications of the altered protein or genomic regulatory region (Chapter 5).

Fitness, replicative capacity, and viral load are directly interconnected parameters, and they

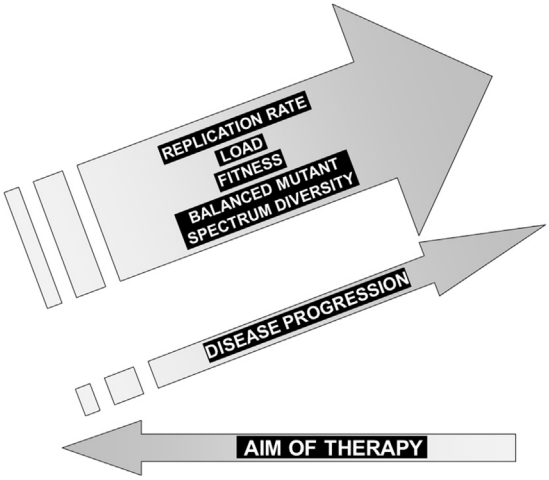

FIGURE 8.9 A schematic representation of interconnected parameters of viral replication that often relate to disease progression. An understanding of quasispecies dynamics has made it evident that the aim of antiviral therapy need be not only to directly diminish the viral load but also to affect other parameters that can then reduce the viral load. See text for justification and references and Chapter 9 for new antiviral strategies that follow the concept expressed in this figure.

affect disease progression (Domingo et al., 2012) (Fig. 8.9). Fitness gain will be more effective with a high load of actively replicating virus in the infected organism. Elevated replicative capacity and fitness sustain high viral loads. The reason for this basic feature of viral population dynamics is that given a basal mutation rate, a large number of replicating genomes entails a correspondingly higher probability that a required mutation for fitness gain can be produced. The events involved are a specific case of search for adaptive mutations in terms of exploration of sequence space, as discussed in Section 3.7 of Chapter 3. While active viral replication, high load, and high fitness favor progression of the infection and disease manifestations, the fourth parameter included in the large arrow of Fig. 8.9, mutant spectrum diversity, has an optimal range. Too low or too high intrapopulation diversity is detrimental to virus adaptability. Insufficient diversity limits adaptability to complex environments (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006), while excess diversity may lead the virus to cross an extinction threshold, and this is the basis of lethal mutagenesis as an antiviral therapy (Chapter 9). An additional implication of the parameters shown in Fig. 8.9 for antiviral interventions is that fitness decrease is recognized as an alternative to inhibition of viral replication to control viral infections[(Clementi, 2008; Clementi and Lazzarin, 2010); reviewed in (Domingo et al. 2012)]. Thus, key features of quasispecies dynamics have a direct implication on the management of viral infections.

8.9 Limitations of simplified reagents and small molecules as antiviral agents

External interventions that have been applied or have been envisaged to limit or suppress virus infection include not only vaccination and administration of antiviral agents as described in previous sections, but also passive immunotherapy, antisense RNAs, or oligonucleotides with various chemical modifications, interfering RNAs, ribozymes, or their combinations. Biotechnological developments have favored the design of chemically defined vaccines (consisting of expressed immunogenic proteins, synthetic peptides, or peptide arrays), without the need to handle or administer live virus. One of the most naïve manifestations of trust in biotechnology in the middle of the 20th century was the belief that a catalog of plasmids encoding the antigenic proteins of the circulating types of pathogenic viruses would suffice to prepare the required vaccine as needed. Concerning influenza vaccines, W.I. Beveridge wrote the following: "The first objective would be to capture the full range of influenza A subtypes. Their antigens would be studied by specialists at central laboratories and made available for the preparation of particular vaccines if and when required. It might be feasible to stockpile some vaccine against all the principal hemagglutinin antigens to be used in a

fire brigade type of action as soon as an incipient pandemic is spotted" (Beveridge, 1977). Naivety is also perceived in current designs of universal vaccines based on conserved antigens, considering the different escape mechanisms that operate in viruses to elude neutralization by antibodies (Chai et al., 2016). It is remarkable how our present understanding of viral populations renders obsolete the views expressed in the W.I. Beveridge book, and in other writings at the boom of implementation of DNA recombinant techniques. Yet, attempts to produce vaccines against highly variable viruses, based on antigenic structures or some viral isolates, are ongoing (Christiansen et al., 2018). A critical issue is if the host immune response against a vaccine engineered with "universal" (conserved) antigenic motifs will be sufficient to prevent disease upon infection by other forms of the same pathogen (Freeman and Cox, 2016). From our current knowledge of viral population dynamics, it seems unlikely but time will tell, since efforts toward the manufacturing of universal antiviral vaccines are under way.

With a conceptual similarity to vaccines, in medical practice, monotherapy with an antiviral agent was traditionally preferred over drug mixtures. (The change of paradigm was largely a consequence of the AIDS epidemic, and it was publicly expressed by the pioneer hepatologist S. Sherlock in a summary address of an International Symposium on Viral Hepatitis held in Madrid in 1998). The change of perspective is clear.

Some antiviral strategies, such as antisense nucleic acids or virus-directed ribozymes, were intensely investigated decades ago. It is unlikely that when used in isolation, they can be converted into useful antiviral therapies because resistant mutants are likely to be selected. Yet, they could be part of combinations with other antiviral inhibitors to provide a larger antiviral barrier (Chapter 9). A similar fate is likely for interfering RNAs (Boden et al., 2003; Gitlin et al., 2005; Herrera-Carrillo and Berkhout, 2015; McDonagh et al., 2015). To a large extent, the failures of defined chemical entities (oligonucleotides, ribozymes, small molecule inhibitors, etc.) to control virus replication and spread are a consequence of their targeting a very defined viral genomic sequence combined with the adaptability of viral populations. Combination of such multiple elements have been envisaged and tested, but off-target effects and the adaptive potential of viruses are likely to limit their efficacy. Unfortunately, biotechnological developments that have been so positive for many research areas and practical applications tend to simplify the types of agents to prevent disease or inhibit virus replication, ignoring the inherent complexity of the object to be controlled. Success is unlikely when "complexity" is combated with "simplicity." An increased understanding of viral population dynamics over the last decades has changed the picture dramatically by providing an interpretation of "virus escape" as a general and largely unavoidable phenomenon. Such awareness has pushed the development of new antiviral designs, which are fundamentally centered in two strategies: a combination of multiple, independently acting elements or fitness decrease through excess mutations (Chapter 9).

8.10 "Hit early, hit hard"

A pronouncement by P. Ehrlich at the International Congress of Medicine held in London in 1913, reflected old traditions on how to treat microbial infections ["Here, therefore, the old therapeutic motto is applicable: *Frapper fort et frapper vitte*" and "Therefore, it is in my opinion necessary to *allow the therapeutic treatment to come into action as early as possible*, ….."; sentences taken from (Ehrlich, 1913), with emphases as in the original text]. This pronouncement fits our current understanding of viral populations. Following Ehrlich, the full implications of quasispecies-mediated adaptation of viruses for

antiviral therapy were expressed by D.D. Ho in an influential article entitled "Time to hit HIV, early and hard" (Ho, 1995). The article title captures what is needed to prevent adaptation of a virus in the infected host. Any opportunity to replicate is exploited by the virus to increase its fitness and to become less vulnerable to internal (intrahost) or external interventions such as antiviral therapy. Treatment interruptions during chronic infections, such as "drug holidays" that in the case of HIV-1-infected patients were justified to alleviate side effects associated with administration of antiretroviral agents, provided an opportunity for the virus to gain fitness. In principle, given our current understanding of viral quasispecies dynamics, the proposal of P. Ehrlich and D.D. Ho is applicable to other viral pathogens.

One argument that tones down the strength of the "hit early and hit hard" proposal is that some infected patients may not progress to disease, but maintain an asymptomatic lifelong persistent infection. This is the case with elite controllers in the case of HIV-1 infection, and individuals infected with HCV who will not progress toward liver disease. In cases in which such nonprogression can be anticipated by viral and host parameters, it may be justified to exclude some patients from aggressive interventions (Suthar and Harries, 2015; Casado et al., 2018). As a general rule, however, the potential benefits of early treatment are obvious not only to avoid disease on an individual basis, but also to diminish the chances of virus transmission (reviewed in Suthar and Harries, 2015; see also Sections 7.1 and 7.7 in Chapter 7 regarding the relevance of viral population numbers in transmission). Restricting the number of treated patients for economic reasons will result in more expensive public health interventions when infected individuals develop the disease.

Box 8.4 includes recommendations for the use of antiviral agents and recapitulates concepts explained in this and preceding sections.

8.11 Information and global action

Despite the emphasis on evolutionary aspects, prevention and treatment of viral disease have many other angles some of which were

BOX 8.4

SOME RECOMMENDATIONS FOR THE USE OF ANTIVIRAL AGENTS

- Avoid monotherapy. Ideally, use two or more antiviral agents which do not share a mechanism of action (Fig. 8.6).
- Treat as soon as possible after virus diagnosis, to avoid virus adaptability associated with high virus population size and to minimize transmission of inhibitor-resistant mutants.
- Individual patients should be treated only during the time at which the drug proves effective. When viral load rebounds, treatment should be discontinued.
- Use deep sequencing methodology to determine mutant spectrum composition for an adequate choice of inhibitors. The aim is to design personalized treatments that consider the probability of drug combination efficacy with minimal side effects.
- Consider temporary shelving of effective drugs when resistant mutants acquire epidemiological relevance.

considered in Chapter 7 in connection with factors of disease emergence (Smolinski et al., 2003). Three of them should be mentioned here because they are as important as the adequate treatment designs described in this chapter: (i) adequacy of public health measures, (ii) public information about virus sources and means of contagion and (iii) need of global political action. Information to the public should aim at limiting the spread of disease that is, undertaking personal and collective actions to reduce the R_0 value for a given virus (Chapter 7, Section 7.2). As an illustration of this key point, there was a quite extensive information campaign on HIV-1 and AIDS in developed countries during the early decades of HIV-1 spread, while the information about other potentially threatening viruses such as Ebola or the severe acute or the Middle East respiratory syndrome (SARS and MERS, respectively) coronaviruses was more limited.

The need of a global response to limit the extension of disease episodes at the sites where they are initiated has been recognized for a long time, but it became obvious with the 2014–15 West African Ebola epidemic (see Siedner et al., 2015). There is a need for international organizations and governments of developed countries to provide the health-care workforce to assist low- and middle-income countries to control viral episodes at an early stage. "Help early, help effectively" is the recognized need at a global scale, which is the parallel to "Hit early, hit hard" for the treatment of infected patients.

Global early action and adequate information can be as important as an adequate treatment design to control viral disease. It can restrict viral replication rounds and consequent adaptability. Information is thought to have been critical for the control of Ebola epidemic in Nigeria (Siedner et al., 2015). However, information must also be planned to reach the target population in a convincing manner, as learned from the poliovirus vaccination and eradication campaign (Renne, 2010). The uncertainties regarding whether an initial, limited episode of viral disease will expand or die out do not help in decision making. However, the best choice in the case of emerging and reemerging infections is to act assuming the worst scenario.

8.12 Overview and concluding remarks

Medical interventions represent a totally new set of selective constraints that viruses are facing only since decades ago, an infinitesimal time of their existence as biological entities. However, the evolutionary mechanisms available to viruses have successfully coped with many selective pressures, notably the effect of vaccination when a broad immune response is not evoked, or treatment with antiviral agents. A common way to proceed is to test a new vaccine with an animal host, be it the authentic host or an animal model, and obtain full protection when the animal is challenged with a virus that matches the antigenic composition of the vaccine. Following the initial excitement, very often the vaccine displays only partial protection when tested in the natural environment. Somewhat parallel arguments can be made about clinical trials for antiviral agents, usually performed initially with selected groups of patients. Clinicians have coined the term "real-life" treatment studies when patients are not selected for optimal results (often pushed by commercial and political interests).

This chapter has emphasized how the complexity of viral populations is a serious (often underestimated) difficulty to prevent and treat viral disease. Examination of the molecular mechanisms exploited by viruses to survive despite antiviral interventions suggests two major lines of action: first, more judicious use of existing tools that should consider the complexity of viral populations and their dynamics; complexity

cannot be combated with simplicity. Second, the need to design new antiviral strategies, a topic addressed in Chapter 9.

Several interconnected parameters determine the probability of success of an antiviral intervention. Most of them follow from the general concepts of Darwinian evolution explained in preceding chapters. It is important, however, to quantify as much as possible the evolutionary events that determine therapy success or failure. For this reason, the importance of viral population size, basic probability calculations of developing resistance, and the selective strength of mutations, have been explained with numerical examples. Hopefully, these simple quantifications will permit a higher awareness of when and why treatment may succeed or fail.

We live in a very unequal society. The chapter closes with the recognition that there are many social economic issues that are as important as scientific planning to combat the pathogenic viruses around us (see Summary Box).

SUMMARY BOX

- Medical interventions represent a new class of selective constraints acting on viral populations.
- Viral evolution affects antiviral preventive and treatment strategies in two different ways: (i) through the molecular mechanisms of short-term response in treated individuals that select escape mutants, and (ii) through the epidemiological impact of viruses that have acquired escape mutations.
- Ineffective vaccines can contribute to the selection of antigenic viral variants.
- Selection of viral mutants resistant to antiviral agents is a general phenomenon. Selection is favored by suboptimal treatments and is delayed by the combined administration of multiple inhibitors. Resistance may also occur in the absence of specific resistance mutations, and it is associated with viral fitness.
- The aim of therapy should be to increase the functional barrier to resistance and to give no opportunity to the virus to pursue replication that increases its replicative fitness.
- Replication rate, viral load, fitness, and mutant spectrum complexity are interconnected parameters that may tip the balance toward either control of the infection or disease progression. Each of these parameters can be targeted in an antiviral design.
- Health care resources, adequate public information, and firm political action are as important as antiviral designs to control virus infections at a global level.

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