CHAPTER

QUASISPECIES DYNAMICS IN DISEASE PREVENTION AND CONTROL

8

CHAPTER CONTENTS

ABBREVIATIONS

Ab antibody
AIDS acquired immunodeficiency syndrome
CC_{50} cytotoxic concentration 50
CTL cytotoxic T cell
FMDV foot-and-mouth disease virus

Virus as Populations. http://dx.doi.org/10.1016/B978-0-12-800837-9.00008-3

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
NGS	next generation sequencing
NNRTIs	nonnucleotide 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 has represented a very powerful external selective constraint imposed upon replicating microbes. Hundreds of antiviral agents have been developed since the second half of the twentieth century, and viruses can generally find evolutionary pathways to continue replication in their presence. The same is true of antibiotic resistance in bacteria. The belief that bacterial diseases were in their way toward extinction was quite widespread in the middle of the twentieth 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 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 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 which 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 large amounts 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. In considering the design of a new antiviral vaccine, the extent of diversity in the field of the virus to be controlled is critical. The natural evolution of the virus may result in the circulation of one major antigenic type or to the co-circulation of multiple antigenic types. 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 should be selected depending on the circulating viruses. This is why anti-FMD vaccines of different composition 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 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 [motivated largely from information obtained by next generation sequencing (NGS) applied to the virus present in each infected patient] has some parallels with vaccine composition design. For vaccines,

the information comes from analyses of antigenic composition of circulating viruses and for antiviral agents, the information comes from the analyses of the quasispecies 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 dramatically decreased as a result of vaccination programs against several viral diseases such as measles or hepatitis B (Bloom and Lambert, 2003), and substantial progress has been made toward the eradication of poliomyelitis (Chumakov and Kew, 2010). 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 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 neuranimidase 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 a vaccine unfeasible at least with the current tools of vaccinology. 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) 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; Hagan et al., 2015). For each virus-host system 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 total absence of replication of the infecting virus (termed "sterilizing" immunity) or absence of disease manifestations despite infection and some 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 which 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 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 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). The 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).

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).

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 obviously 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. The above abstraction reflects, however, 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 epitopic repertoire to the immune system. 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). 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.

8.3.2 VACCINATION-INDUCED EVOLUTION

If a virus is allowed to circulate in a population where vaccinated and unvaccinated host individuals coexist, if the vaccine does not induce sterilizing immunity as is often the case, viruses with an altered antigenic profile might be gradually selected. 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 zoonotic transmission of viruses 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, reviews in Gandon et al., 2003; Schat and Baranowski, 2007). The timing of dominance of CTL-escape mutants of 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 one 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).

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, insufficient time between vaccination and exposure to the viral pathogen, and antigenic differences between the vaccine strains and circulating viruses. In addition, for massive vaccinations in veterinary medicine, damage to the vaccine and improper administration are additional problems. Vaccination may occasionally promote the selection not only of antigenic variants but also of host cell tropism, host range or virulent variants (Swayne and Kapczynski, 2008; Kirkwood, 2010; Read et al. 2015). To what extent the widespread use of vaccination can contribute to antigenic variation relative to other factors (genetic drift due to genetic bottlenecks, etc.) is not known. 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.

The above observations with animal and human viruses suggest the following possible scenarios. 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 1 per 500,000 to 1 per 750,000 vaccinees, and those receiving the second vaccine dose the rate was about 1 in 12 million (reviewed in Rowlands and Minor, 2010). Attenuated anti-FMD vaccines were used in some countries during the second half of the twentieth century, but reversion to virulence forced halting 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 co-infecting related viruses, as observed with poliovirus and bovine herpesvirus-1 [Kew et al., 2002; Thiry et al., 2006; among other studies with these and additional viruses]. Reiteration of vaccine selection and fitness increase processes over many generations of vaccinees (be 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 enzymological 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 similarly 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).

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. 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 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 descriptions for specific groups of viruses) [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, 2010; the 10 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 type of mutation associated with a phenotypic change) that is generated at a frequency of 10^{-4} or lower will be present in 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) (Figure 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 Figure 8.1

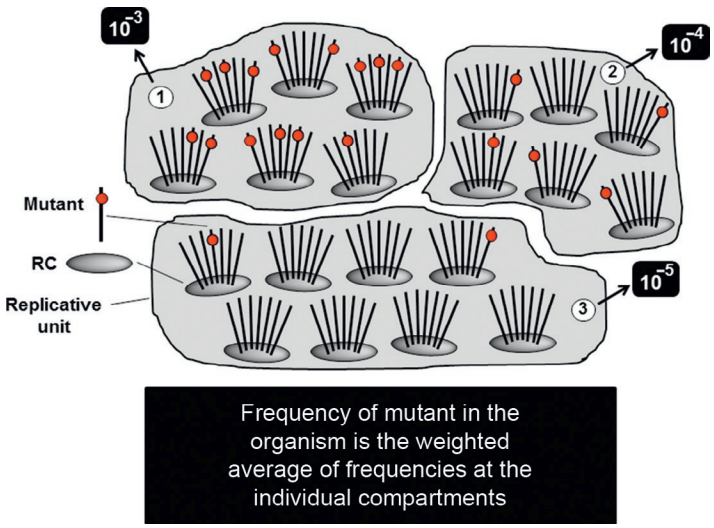

FIGURE 8.1

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 3 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.

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 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 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 to other compartments.

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 individual 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 Figures 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.

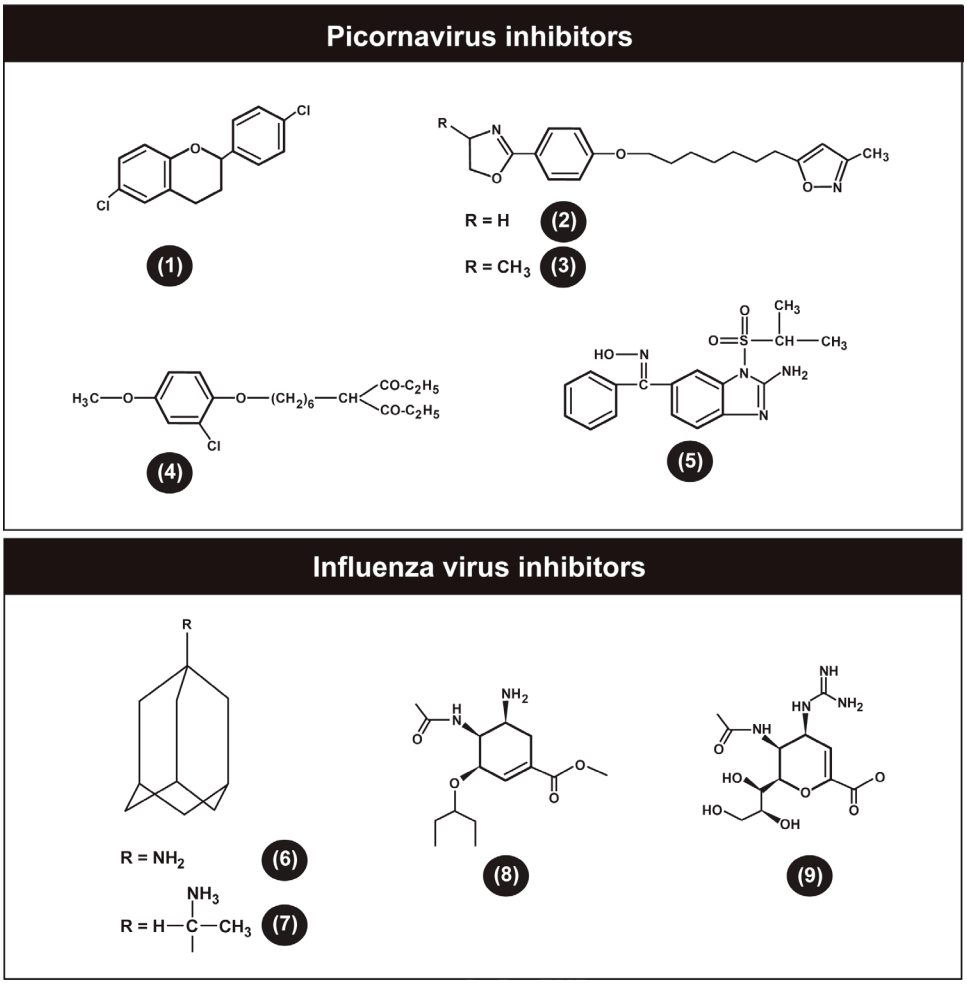

FIGURE 8.2

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 51711). (3) WIN 52084. (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.

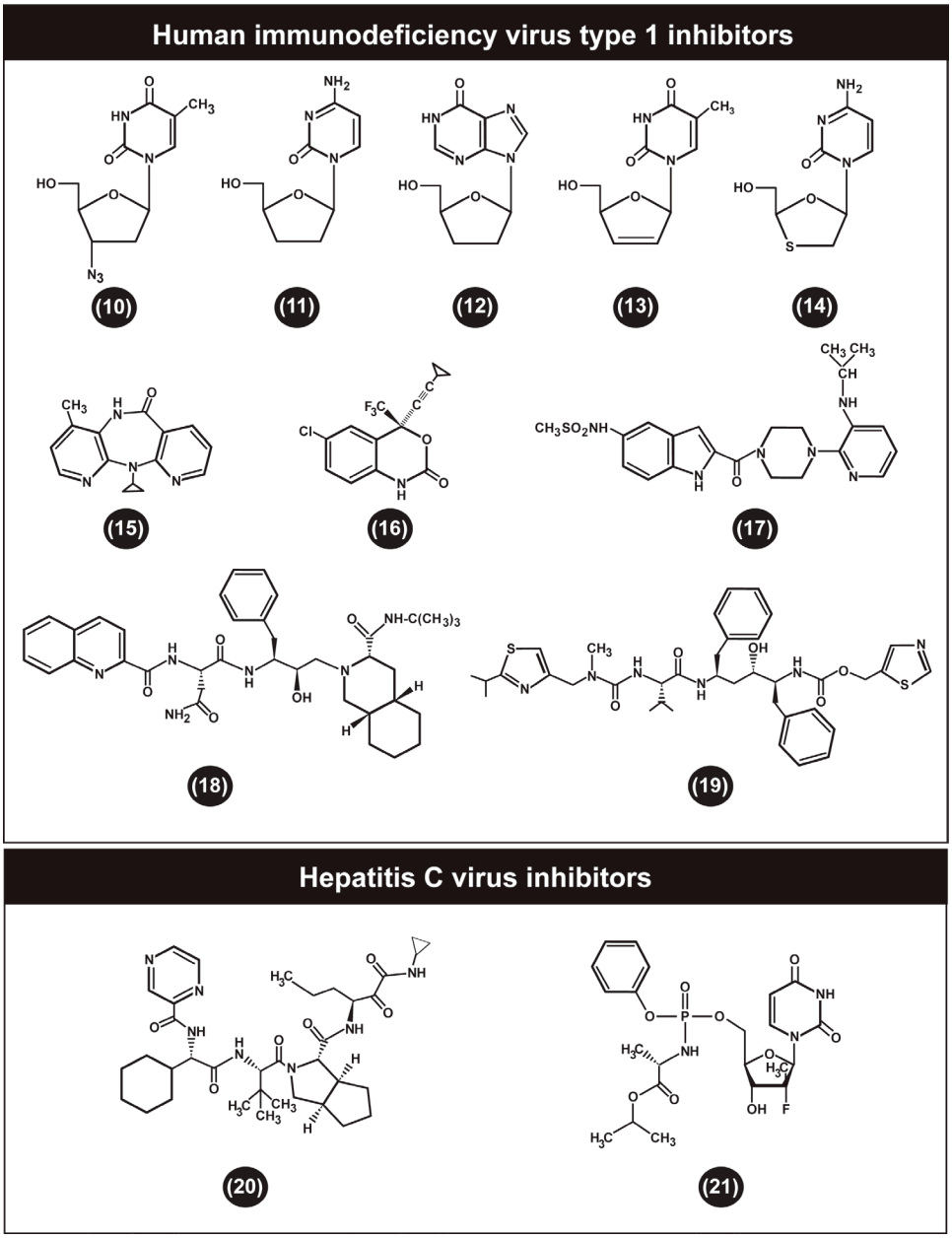

FIGURE 8.3

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.

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 Figure 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 evolutionary outcome). The requirement of transitions versus transversions will affect the genetic barrier to antiviral resistance. Most viral polymerases tend to produce transition mutations more readily than transversions presumably because in the course

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 dominance of resistant mutants.
- The combined effect of the different barrier classes determine the ease of dominance of drug-resistant mutants in populations subjected to the selective pressure of one or multiple inhibitors.

FIGURE 8.3–CONT'D

(14) Lamivudine (3TC), 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[[[(2*R*,3*R*,4*R*,5*R*)-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.

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. To complicate matters even more, since in many cases several independent mutations may confer resistance to the same drug, it has to be considered also 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) as it may affect 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 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 selection of escape mutants. Sofosbuvir [formula (21) in Figure 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 NGS 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 true reversion of mutant genomes rather than outgrowth of baseline wild-type genomes (Hedskog et al., 2015). This result suggests a high phenotypic barrier for sofosbuvir, but that HCV has mechanisms to overcome this barrier. The complexities of virus-host interactions render the elucidation on the pathways exploited by a virus to overcome the phenotypic barrier to a drug a highly empirical endeavor. The hope is that by combining high phenotypic barrier inhibitors, the forced reversion of the resistance mutations for survival may 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). The possible contribution of mutant swarms to facilitate or impede 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 particular target organ, at a given time. Additional influences are drug pharmacokinetics, drug penetration into different cells, tissues, and organs where the virus replicates (see Figure 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

The genetic barrier, as defined in Box 8.3, can be anticipated from the number of point mutations that, according to the genetic code, are needed to convert an amino acid associated with drug sensitivity into another amino acid that confers drug resistance. 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 Figure 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 Figure 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 be also applied to cell culture measurements.

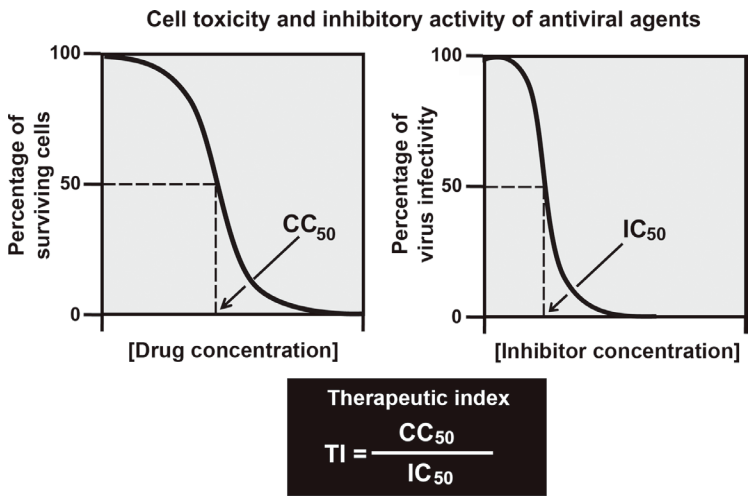

FIGURE 8.4

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 influences, as repeatedly expressed for other features of viruses in the present book. As a guide, TI values of 100 or more suggest excellent performance of an antiviral agent, values higher than 10 are acceptable, but values lower than 10 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 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 (Figure 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 gradually dominant and can be isolated for further studies. To give another example, a single amino acid

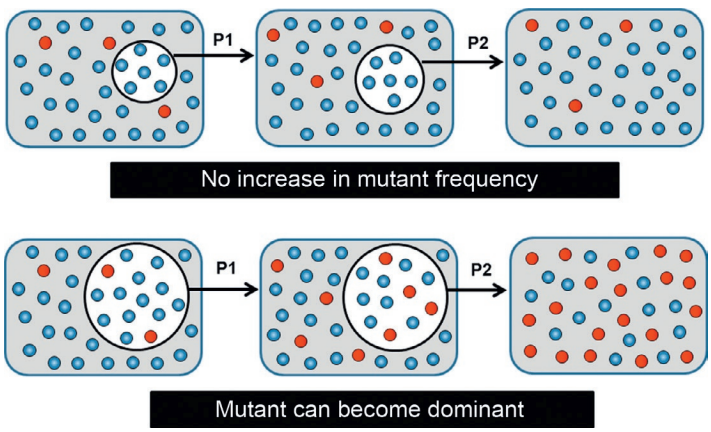

FIGURE 8.5

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.

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 lower frequency than replacements that require a single mutation. If each of the two mutations reach a frequency of 2×10^{-4}, the expected frequency of the drug-resistant genomes (ignoring fitness effects) will be $(2\times10^{-4})\times(2\times10^{-4})=4\times10^{-8}$. 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 a process of positive selection (compare with Section 3.2 and Figure 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 Figure 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 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 (Figure 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

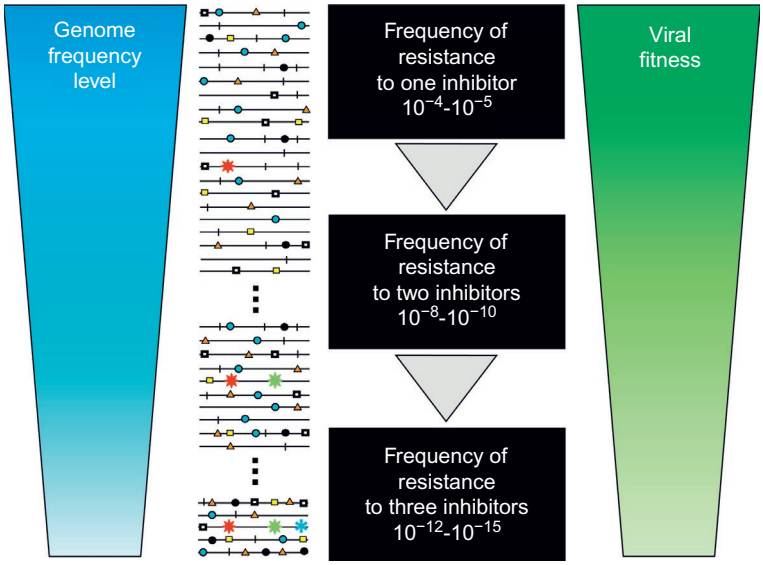

FIGURE 8.6

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.

experiment was designed to infect with an insufficient amount of virus. Similar, even more accentuated problems are encountered in selection experiments *in vivo*, since not only population size but also the source of the virus (blood and other organ) for the next infection may determine the inclusion or exclusion of the relevant mutation (compare Figures 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 wild-type amino acid (the one that confers drug sensitivity) relative to the virus expressing the substitution 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 Figure 8.7; the assays are best performed in cell culture, although use of explants or *in vivo* assays are 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, and the selective advantage conferred by the substitution in the presence of the drug. In the presence of the drug, the mutant will yield a fitness value $f_{+DRUG} > 1$ relative to the wild type (necessarily if the mutation confers resistance and virus viability is preserved). In the absence of the drug, $f_{-DRUG} \leq 1$ relative to the wild type quantifies the fitness cost of the resistance mutation; the lower the value of f_{-DRUG}, the higher the fitness cost. We define the selective strength of the resistance mutation as f_{+DRUG}/f_{-DRUG}. For example, if we put arbitrary numbers (unrelated to values shown in ordinate) to the fitness values in the first graph of Figure 8.7, $f_{+DRUG} = 1.4$ and $f_{-DRUG} = 0.8$, we obtain a selective

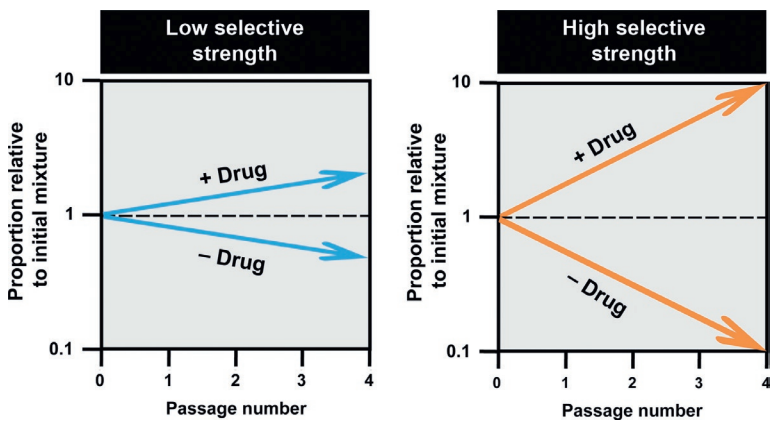

FIGURE 8.7

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 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.

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 the 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. 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 (not to say never) 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 finding compensatory mutations. Not only the connectivity among points of sequence space plays a role but also the fact that several points in sequence space map into the same (or similar) drug-resistance phenotype. 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 detail 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 (Figures 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 viral disease.

8.5 MOLECULAR MECHANISMS OF ANTIVIRAL RESISTANCE

The great majority of inhibitor-resistance mechanisms involve amino acid substitutions in viral proteins. The substitutions may render the drug ineffective through the following mechanisms:

- Substitutions in the protein targeted by the drug that decrease the affinity of the protein for the drug. To this group belong mutations that modify nucleotide selectivity in viral polymerases.
- 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 Figure 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 in 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 in use or under preclinical development (reviews in Menendez-Arias, 2010, 2013; for a predictive model that includes nucleotide levels, see von Kleist et al., 2012).

Nonnucleotide RT inhibitors (NNRTIs) bind to a 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 Figure 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 (Menendez-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 Figure 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 Menendez-Arias (2010 and 2013) provide excellent and detailed accounts of mechanisms of resistance to HIV-1 inhibitors that include virus entry and integrase inhibitors in addition to the RT and protease inhibitor briefly described here. Despite variations in the detailed mechanisms involved, resistance to inhibitors of other major viral pathogens such as HCV and HBV is a general phenomenon, and the number of resistance mutations keeps increasing as new treatments become available.

8.5.2 MUTATION SITE AND FUNCTIONAL BARRIER

Because of the distance effects that can be exerted among amino acids in viral 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. When an enzymological 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 the new directly 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, decreased 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 are less likely to 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 a frequent range is 10^{-4} to 10^{-6} mutants per infectious unit 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 with frequency measurements. A point worth emphasizing is that the quantification of viruses harboring biologically relevant mutations has been possible because an adequate biological

Table 8.1 The Frequency of Some Drug-Escape Mutants

Drug[a]	Virus[b]	Frequency	Comments	Reference
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 1 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]Guanidinium is carbamimidoylazanium.

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 on average more prone to variation than other amino acids in viral proteins. This means that if we had additional selective agents to probe other viral sites, we would probably obtain 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).

The calculated frequencies of resistant mutants in viral populations may be lower than the rate at which they originate by mutation due to fitness cost of the mutation (Section 8.4.4). The argument is parallel to the one used to justify why mutation rates and frequencies differ due to 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. In other words, the basal level of mutational pressure may be sufficient to provide a detectable level of escape mutants without the need of selection by the selective agent. This is an important aspect of antiviral therapy which 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 of 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 (Havlir et al., 1996; Lech et al., 1996; Cubero et al., 2008; Johnson et al., 2008; Toni et al., 2009; Tsibris et al., 2009, among other studies). Ample support has come also from NGS 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. 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.

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. Despite being the most common, the presence of specific resistance mutations is not the only mechanism of drug resistance. The cell culture system of HCV replication in human hepatoma cells (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) permitted 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 directly acting antiviral 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 due to 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 Figure 8.3], the NS5A inhibitor daclatasvir, the cellular protein cyclophilin A inhibitor cyclosporin A, and the mutagenic purine nucleoside ribavirin. HCVp45 and HCVp100 displayed significant increased resistance to all inhibitors tested, as compared with the parental population HCVp0 (Sheldon et al., 2014) (Figure 8.8). Passage of HCV entailed an increase of viral fitness and a broadening of the mutant spectrum, as expected (Section 5.4 of Chapter 5). Therefore, a clear possibility was that the broadening of the mutant spectrum 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 NGS 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 (Figure 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 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 with their corresponding parental, uncloned populations (Sheldon et al., 2014).

The above observations have established viral fitness, or some trait associated with viral fitness, as a multidrug-resistance determinant in HCV, that may also apply to other viruses. One possible molecular mechanism may consist in a 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 in the number of inhibitor molecules that reach the replication sites. Exploration of this model and other possible models, and the extension to other viral-host systems, are important challenges in the field of antiviral research.

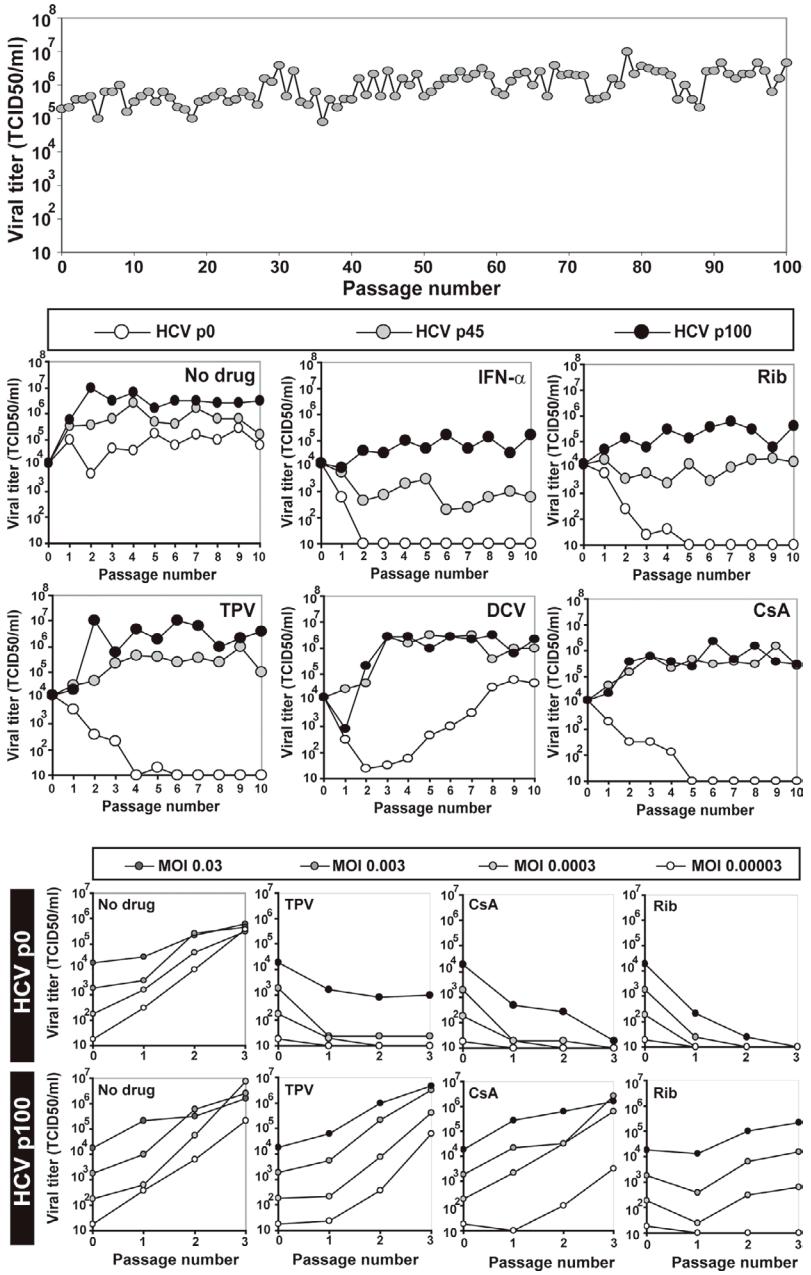

FIGURE 8.8

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 et al. (2014), with permission from the American Society for Microbiology, Washington DC, USA.

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; Sato et al., 2015). 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 of 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, an efficient early control of virus replication by the host immune response is generally associated with limited disease severity. In HIV-1, the viral load that follows after the initial immune response to the virus is referred to as the "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 host and viral factors. [This and other aspects of HIV-1 replication and pathogenesis have been reviewed in excellent monographs by Levy (2007)]. A low set point predicts an asymptomatic outcome, and this is generally the case for viruses that establish persistent rather than acute infections. Again, viral population numbers, derived from the virus-host interaction, play a critical role in the result of an infection. Given a host environment, an initial high viral fitness during the early stages of viral replication can promote disease manifestations. An example was provided by the progression toward disease of a cohort of individuals that were infected during blood transfusion with an HIV-1 containing a large deletion in Nef. After more than 15 years, some of the infected individuals showed clinical signs, probably as a result of accumulation of mutations in the HIV-1 genome that compensated for the lack of Nef, an adaptor protein that mediates replication and pathogenesis (reviewed in Arien and Verhasselt, 2008). 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 affect disease progression (Domingo et al., 2012) (Figure 8.9). Fitness gain will be more effective, the higher is the load of actively replicating virus in the infected organism. High 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 Figure 8.9, mutant spectrum diversity, has an optimal range. Too low or too high intrapopulation diversity is detrimental

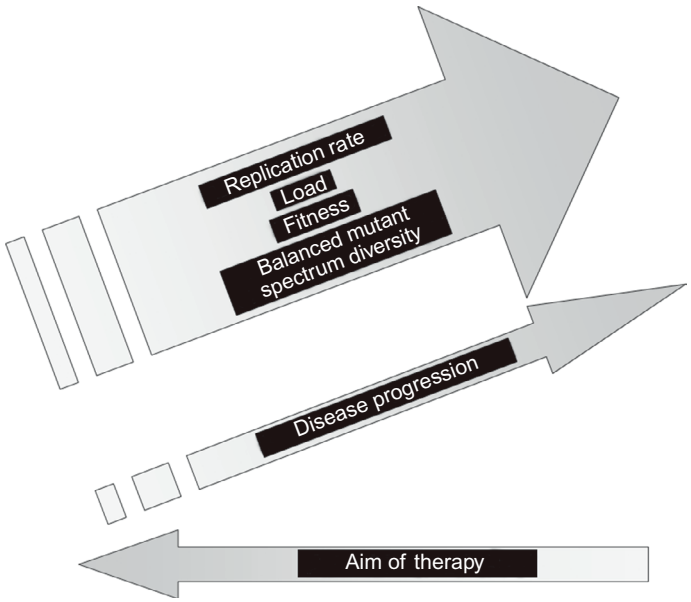

FIGURE 8.9

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 not be only to diminish directly the viral load, but to affect other parameters that then can 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.

to virus adaptability. Insufficient diversity limits adaptability to complex environments (Pfeiffer and Kirkegaard, 2005; Vignuzzi et al., 2006), while excess diversity may force 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 Figure 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 are 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 naive manifestations of trust in biotechnology in the middle of the twentieth century was the belief that a catalog of plasmids encoding the antigenic proteins of the circulating types of a pathogenic virus 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. It is remarkable how our present perception of viral populations differs from the views expressed in the W.I. Beveridge book and in other writings at the boom of implementation of DNA recombinant techniques. With a conceptual similarity, 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 multiple such 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 an awareness has pushed the development of new antiviral designs, which are fundamentally centered in two strategies: combination of multiple, independently acting elements or fitness decrease through excess mutations (Chapter 9).

8.10 "HIT EARLY, HIT HARD"

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

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 (Figure 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 NGS methodology to determine mutant spectrum composition for adequate choice of inhibitors. The aim is to design personalized treatments that consider probability of drug combination efficacy with minimal side effects.
- Consider temporary shelving of effective drugs when resistant mutants acquire epidemiological relevance.

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 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 the 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). 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 the infected individuals develop 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 emphasis on evolutionary aspects, prevention and treatment of viral disease has many other angles some of which were considered in Chapter 7 in connection with factors of disease emergence (Smolinski et al., 2003). Two of them should be mentioned here because they are as important as the adequate treatment designs described in this chapter: (i) public information about virus sources and means of contagion and (ii) need of global actions. 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 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 2014-2015 West African Ebola epidemic (see Siedner et al., 2015). There is a need for international organizations and governments of developed countries to provide health-care work force 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 be also planned to reach the target population in a convincing manner, as learnt 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 does 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. Yet, the evolutionary mechanisms available to viruses have successfully coped with many selective pressures, notably vaccines that do not evoke a broad immune response or treatment with antiviral agents. A common scenario with obvious commercial and sometimes even political influences 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. This chapter has attempted to emphasize 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 persist despite interventions suggests two major lines of action: first, a 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 analyzed with numerical examples. Hopefully, these simple quantifications will permit a higher awareness of when and why a 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: through the long-term diversification of viruses in nature, and through the molecular mechanisms of short-term response in treated individuals.
- Ineffective vaccines can contribute to 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 or a fitness-related trait.
- 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.
- Firm political action and adequate public information is as important as antiviral designs to control virus infections at a global level.

REFERENCES

Appleyard, G., 1977. Amantadine-resistance as a genetic marker for influenza viruses. J. Gen. Virol. 36, 249–255.

Arien, K.K., Verhasselt, B., 2008. HIV Nef: role in pathogenesis and viral fitness. Curr. HIV Res. 6, 200–208.

Bailey, J., Blankson, J.N., Wind-Rotolo, M., Siliciano, R.F., 2004. Mechanisms of HIV-1 escape from immune responses and antiretroviral drugs. Curr. Opin. Immunol. 16, 470–476.

Beerenwinkel, N., Daumer, M., Sing, T., Rahnenfuhrer, J., Lengauer, T., et al., 2005. Estimating HIV evolutionary pathways and the genetic barrier to drug resistance. J. Infect. Dis. 191, 1953–1960.

Belshe, R.B., Smith, M.H., Hall, C.B., Betts, R., Hay, A.J., 1988. Genetic basis of resistance to rimantadine emerging during treatment of influenza virus infection. J. Virol. 62, 1508–1512.

Beveridge, W.I.B., 1977. Influenza: The Last Great Plague; and Unfinished Story of Discovery. Prodist, New York.

Bloom, B.R., Lambert, P.-H., 2003. The Vaccine Book. Academic Press, Elsevier, San Diego.

Boden, D., Pusch, O., Lee, F., Tucker, L., Ramratnam, B., 2003. Human immunodeficiency virus type 1 escape from RNA interference. J. Virol. 77, 11531–11535.

Burnet, M., 1966. Natural History of Infections Disease. Cambridge University Press, London.

Cale, E.M., Hraber, P., Giorgi, E.E., Fischer, W., Bhattacharya, T., et al., 2011. Epitope-specific CD8+ T lymphocytes cross-recognize mutant simian immunodeficiency virus (SIV) sequences but fail to contain very early evolution and eventual fixation of epitope escape mutations during SIV infection. J. Virol. 85, 3746–3757.

Cheng, B.Y., Ortiz-Riano, E., Nogales, A., de la Torre, J.C., Martinez-Sobrido, L., 2015. Development of live-attenuated arenavirus vaccines based on codon deoptimization. J. Virol. 89, 3523–3533.

Chumakov, K., Kew, O., 2010. The poliovirus eradication initiative. In: Ehrenfeld, E., Domingo, E., Roos, R.P. (Eds.), The Picornaviruses. ASM Press, Washington, DC, pp. 449–459.

Ciurea, A., Klenerman, P., Hunziker, L., Horvath, E., Senn, B.M., et al., 2000. Viral persistence *in vivo* through selection of neutralizing antibody- escape variants. Proc. Natl. Acad. Sci. U. S. A. 97, 2749–2754.

Ciurea, A., Hunziker, L., Martinic, M.M., Oxenius, A., Hengartner, H., et al., 2001. CD4+ T-cell-epitope escape mutant virus selected in vivo. Nat. Med. 7, 795–800.

Clementi, M., 2008. Perspectives and opportunities for novel antiviral treatments targeting virus fitness. Clin. Microbiol. Infect. 14, 629–631.

Clementi, M., Lazzarin, A., 2010. Human immunodeficiency virus type 1 fitness and tropism: concept, quantification, and clinical relevance. Clin. Microbiol. Infect. 16, 1532–1538.

Coleman, J.R., Papamichail, D., Skiena, S., Futcher, B., Wimmer, E., et al., 2008. Virus attenuation by genome-scale changes in codon pair bias. Science 320, 1784–1787.

Crowder, S., Kirkegaard, K., 2005. Trans-dominant inhibition of RNA viral replication can slow growth of drug-resistant viruses. Nat. Genet. 37, 701–709.

Cubero, M., Esteban, J.I., Otero, T., Sauleda, S., Bes, M., et al., 2008. Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. Virology 370, 237–245.

Domingo, E., 1989. RNA virus evolution and the control of viral disease. Prog. Drug Res. 33, 93–133.

Domingo, E., Holland, J.J., 1992. Complications of RNA heterogeneity for the engineering of virus vaccines and antiviral agents. Genet. Eng. (N.Y.) 14, 13–31.

Domingo, E., Biebricher, C., Eigen, M., Holland, J.J., 2001a. Quasispecies and RNA Virus Evolution: Principles and Consequences. Landes Bioscience, Austin.

Domingo, E., Mas, A., Yuste, E., Pariente, N., Sierra, S., et al., 2001b. Virus population dynamics, fitness variations and the control of viral disease: an update. Prog. Drug Res. 57, 77–115.

Domingo, E., Sheldon, J., Perales, C., 2012. Viral quasispecies evolution. Microbiol. Mol. Biol. Rev. 76, 159–216.

Eggers, H.J., Tamm, I., 1961. Spectrum and characteristics of the virus inhibitory action of 2-(alpha-hydroxybenzyl)-benzimidazole. J. Exp. Med. 113, 657–682.

Eggers, H.J., Tamm, I., 1965. Coxsackie A9 virus: mutation from drug dependence to drug independence. Science 148, 97–98.

Evans, A.S., Kaslow, R.A., 1997. Viral Infections of Humans. Epidemiology and Control. Plenum Medical Book Company, New York and London.

Fischer, W., Ganusov, V.V., Giorgi, E.E., Hraber, P.T., Keele, B.F., et al., 2010. Transmission of single HIV-1 genomes and dynamics of early immune escape revealed by ultra-deep sequencing. PLoS One 5, e12303.

Flynn, W.F., Chang, M.W., Tan, Z., Oliveira, G., Yuan, J., et al., 2015. Deep sequencing of protease inhibitor resistant HIV patient isolates reveals patterns of correlated mutations in gag and protease. PLoS Comput. Biol. 11, e1004249.

Fun, A., Wensing, A.M., Verheyen, J., Nijhuis, M., 2012. Human immunodeficiency virus gag and protease: partners in resistance. Retrovirology 9, 63.

Gandon, S., Mackinnon, M., Nee, S., Read, A., 2003. Imperfect vaccination: some epidemiological and evolutionary consequences. Proc. Biol. Sci. 270, 1129–1136.

Gitlin, L., Stone, J.K., Andino, R., 2005. Poliovirus escape from RNA interference: short interfering RNA-target recognition and implications for therapeutic approaches. J. Virol. 79, 1027–1035.

Golan, D.E., Tashjian, A.H. (Eds.), 2011. Principles of Pharmacology: The Pathophysiologic Basis of Drug Therapy. Lippincott Williams & Wilkins, Baltimore, Philadelphia.

Hagan, T., Nakaya, H.I., Subramaniam, S., Pulendran, B., 2015. Systems vaccinology: enabling rational vaccine design with systems biological approaches. Vaccine, in press.

Havlir, D.V., Eastman, S., Gamst, A., Richman, D.D., 1996. Nevirapine-resistant human immunodeficiency virus: kinetics of replication and estimated prevalence in untreated patients. J. Virol. 70, 7894–7899.

Hedskog, C., Dvory-Sobol, H., Gontcharova, V., Martin, R., Ouyang, W., et al., 2015. Evolution of the HCV viral population from a patient with S282T detected at relapse after sofosbuvir monotherapy. J. Viral Hepat., in press.

Heinz, B.A., Rueckert, R.R., Shepard, D.A., Dutko, F.J., McKinlay, M.A., et al., 1989. Genetic and molecular analyses of spontaneous mutants of human rhinovirus 14 that are resistant to an antiviral compound. J. Virol. 63, 2476–2485.

Herrera-Carrillo, E., Berkhout, B., 2015. The impact of HIV-1 genetic diversity on the efficacy of a combinatorial RNAi-based gene therapy. Gene Ther. 22, 485–495.
Herrmann Jr., E.C., Herrmann, J.A., 1977. A working hypothesis–virus resistance development as an indicator of specific antiviral activity. Ann. N. Y. Acad. Sci. 284, 632–637.
Ho, D.D., 1995. Time to hit HIV, early and hard. N. Engl. J. Med. 333, 450–451.
Hsu, H.Y., Chang, M.H., Liaw, S.H., Ni, Y.H., Chen, H.L., 1999. Changes of hepatitis B surface antigen variants in carrier children before and after universal vaccination in Taiwan. Hepatology 30, 1312–1317.
Ji, W., Niu, D.D., Si, H.L., Ding, N.Z., He, C.Q., 2014. Vaccination influences the evolution of classical swine fever virus. Infect. Genet. Evol. 25, 69–77.
Johnson, J.A., Li, J.F., Wei, X., Lipscomb, J., Irlbeck, D., et al., 2008. Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. PLoS Med. 5, e158.
Kekarainen, T., Gonzalez, A., Llorens, A., Segales, J., 2014. Genetic variability of porcine circovirus 2 in vaccinating and non-vaccinating commercial farms. J. Gen. Virol. 95, 1734–1742.
Kew, O., Morris-Glasgow, V., Landaverde, M., Burns, C., Shaw, J., et al., 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science 296, 356–359.
Kilbourne, E.D., 1987. Influenza. Plenum Medical Book Company, New York.
Kirkwood, C.D., 2010. Genetic and antigenic diversity of human rotaviruses: potential impact on vaccination programs. J. Infect. Dis. 202 (Suppl), S43–S48.
Kortenhoeven, C., Joubert, F., Bastos, A., Abolnik, C., 2015. Virus genome dynamics under different propagation pressures: reconstruction of whole genome haplotypes of west nile viruses from NGS data. BMC Genomics 16, 118.
Lech, W.J., Wang, G., Yang, Y.L., Chee, Y., Dorman, K., et al., 1996. In vivo sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects. J. Virol. 70, 2038–2043.
Levy, J.A., 2007. HIV and the Pathogenesis of AIDS. ASM Press, Whashington, DC.
Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., et al., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.
Loh, L., Petravic, J., Batten, C.J., Davenport, M.P., Kent, S.J., 2008. Vaccination and timing influence SIV immune escape viral dynamics in vivo. PLoS Pathog. 4, e12.
Lubeck, M.D., Schulman, J.L., Palese, P., 1978. Susceptibility of influenza A viruses to amantadine is influenced by the gene coding for M protein. J. Virol. 28, 710–716.
Margeridon-Thermet, S., Shafer, R.W., 2010. Comparison of the mechanisms of drug resistance among HIV, hepatitis B, and hepatitis C. Viruses 2, 2696–2739.
Martinez-Picado, J., Martinez, M.A., 2008. HIV-1 reverse transcriptase inhibitor resistance mutations and fitness: a view from the clinic and ex vivo. Virus Res. 134, 104–123.
McDonagh, P., Sheehy, P.A., Norris, J.M., 2015. Combination siRNA therapy against feline coronavirus can delay the emergence of antiviral resistance in vitro. Vet. Microbiol. 176, 10–18.
Melnick, J.L., Crowther, D., Barrera-Oro, J., 1961. Rapid development of drug-resistant mutants of poliovirus. Science 134, 557.
Menendez-Arias, L., 2010. Molecular basis of human immunodeficiency virus drug resistance: an update. Antiviral Res. 85, 210–231.
Menendez-Arias, L., 2013. Molecular basis of human immunodeficiency virus type 1 drug resistance: overview and recent developments. Antiviral Res. 98, 93–120.
Menendez-Arias, L., Richman, D.D., 2014. Editorial overview: antivirals and resistance: advances and challenges ahead. Curr. Opin. Virol. 8, iv–vii.
Mitchison, D.A., 1950. Development of streptomycin resistant strains of tubercle bacilli in pulmonary tuberculosis; results of simultaneous sensitivity tests in liquid and on solid media. Thorax 5, 144–161.
Mohri, H., Singh, M.K., Ching, W.T., Ho, D.D., 1993. Quantitation of zidovudine-resistant human immunodeficiency virus type 1 in the blood of treated and untreated patients. Proc. Natl. Acad. Sci. U. S. A. 90, 25–29.

Muylkens, B., Meurens, F., Schynts, F., Farnir, F., Pourchet, A., et al., 2006. Intraspecific bovine herpesvirus 1 recombinants carrying glycoprotein E deletion as a vaccine marker are virulent in cattle. J. Gen. Virol. 87, 2149–2154.

Nájera, I., Richman, D.D., Olivares, I., Rojas, J.M., Peinado, M.A., et al., 1994. Natural occurrence of drug resistance mutations in the reverse transcriptase of human immunodeficiency virus type 1 isolates. AIDS Res. Hum. Retroviruses 10, 1479–1488.

Nájera, I., Holguín, A., Quiñones-Mateu, M.E., Muñoz-Fernández, M.A., Nájera, R., et al., 1995. Pol gene quasispecies of human immunodeficiency virus: mutations associated with drug resistance in virus from patients undergoing no drug therapy. J. Virol. 69, 23–31.

Pawlotsky, J.M., 2006. Hepatitis C virus population dynamics during infection. Curr. Top. Microbiol. Immunol. 299, 261–284.

Perales, C., Agudo, R., Manrubia, S.C., Domingo, E., 2011. Influence of mutagenesis and viral load on the sustained low-level replication of an RNA virus. J. Mol. Biol. 407, 60–78.

Perales, C., Beach, N.M., Gallego, I., Soria, M.E., Quer, J., et al., 2013. Response of hepatitis C virus to long-term passage in the presence of alpha interferon: multiple mutations and a common phenotype. J. Virol. 87, 7593–7607.

Perales, C., Beach, N.M., Sheldon, J., Domingo, E., 2014. Molecular basis of interferon resistance in hepatitis C virus. Curr. Opin. Virol. 8, 38–44.

Perez-Sautu, U., Costafreda, M.I., Cayla, J., Tortajada, C., Lite, J., et al., 2011. Hepatitis a virus vaccine escape variants and potential new serotype emergence. Emerg. Infect. Dis. 17, 734–737.

Pfeiffer, J.K., Kirkegaard, K., 2005. Increased fidelity reduces poliovirus fitness under selective pressure in mice. PLoS Pathog. 1, 102–110.

Pincus, S.E., Wimmer, E., 1986. Production of guanidine-resistant and -dependent poliovirus mutants from cloned cDNA: mutations in polypeptide 2C are directly responsible for altered guanidine sensitivity. J. Virol. 60, 793–796.

Pircher, H., Moskophidis, D., Rohrer, U., Burki, K., Hengartner, H., et al., 1990. Viral escape by selection of cytotoxic T cell-resistant virus variants *in vivo*. Nature 346, 629–633.

Read, A.F., Baigent, S.J., Powers, C., Kgosana, L.B., Blackwell, L., et al., 2015. Imperfect vaccination can enhance the transmission of highly virulent pathogens. PLoS Biol. 13 (7): e1002198.

Renne, E.P., 2010. The Politics of Polio in Northern Nigeria. Indiana University Press, Bloomington.

Rhee, S.Y., Taylor, J., Fessel, W.J., Kaufman, D., Towner, W., et al., 2010. HIV-1 protease mutations and protease inhibitor cross-resistance. Antimicrob. Agents Chemother. 54, 4253–4261.

Ribeiro, R.M., Bonhoeffer, S., 2000. Production of resistant HIV mutants during antiretroviral therapy. Proc. Natl. Acad. Sci. U. S. A. 97, 7681–7686.

Richman, D.D., 1994. Resistance, drug failure, and disease progression. AIDS Res. Hum. Retroviruses 10, 901–905.

Richman, D.D. (Ed.), 1996. Antiviral Drug Resistance. John Wiley and Sons Inc., New York.

Richman, D.D., Wrin, T., Little, S.J., Petropoulos, C.J., 2003. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc. Natl. Acad. Sci. U. S. A. 100, 4144–4149.

Rowlands, D.J., Minor, P.D., 2010. Vaccine strategies. In: Ehrenfeld, E., Domingo, E., Roos, R.P. (Eds.), The Picornaviruses. ASM Press, Washington, DC, pp. 431–448.

Sarrazin, C., Zeuzem, S., 2010. Resistance to direct antiviral agents in patients with hepatitis C virus infection. Gastroenterology 138, 447–462.

Sato, M., Maekawa, S., Komatsu, N., Tatsumi, A., Miura, M., et al., 2015. Deep Sequencing and phylogenetic analysis of variants resistant to interferon-based protease inhibitor therapy in chronic hepatitis induced by genotype-1b hepatitis C virus. J. Virol. 89: 6105–6116.

Schat, K.A., Baranowski, E., 2007. Animal vaccination and the evolution of viral pathogens. Rev. Sci. Tech. 26, 327–338.

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.

Siedner, M.J., Gostin, L.O., Cranmer, H.H., Kraemer, J.D., 2015. Strengthening the detection of and early response to public health emergencies: lessons from the West African Ebola epidemic. PLoS Med. 12, e1001804.

Smolinski, M.S., Hamburg, M.A., Lederberg, J. (Eds.), 2003. Microbial Threats to Health. Emergence, Detection and Response. The National Academies Press, Washington DC.

Sullivan, J.C., De Meyer, S., Bartels, D.J., Dierynck, I., Zhang, E.Z., et al., 2013. Evolution of treatment-emergent resistant variants in telaprevir phase 3 clinical trials. Clin. Infect. Dis. 57, 221–229.

Suthar, A.B., Harries, A.D., 2015. A public health approach to hepatitis C control in low- and middle-income countries. PLoS Med. 12, e1001795.

Swayne, D.E., Kapczynski, D., 2008. Strategies and challenges for eliciting immunity against avian influenza virus in birds. Immunol. Rev. 225, 314–331.

Taboga, O., Tami, C., Carrillo, E., Núñez, J.I., Rodríguez, A., et al., 1997. A large-scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. J. Virol. 71, 2606–2614.

Tami, C., Taboga, O., Berinstein, A., Nuñez, J.I., Palma, E.L., et al., 2003. Evidence of the coevolution of antigenicity and host cell tropism of foot-and-mouth disease virus in vivo. J. Virol. 77, 1219–1226.

Thiry, E., Muylkens, B., Meurens, F., Gogev, S., Thiry, J., et al., 2006. Recombination in the alphaherpesvirus bovine herpesvirus 1. Vet. Microbiol. 113, 171–177.

Toni, T.A., Asahchop, E.L., Moisi, D., Ntemgwa, M., Oliveira, M., et al., 2009. Detection of human immunodeficiency virus (HIV) type 1 M184V and K103N minority variants in patients with primary HIV infection. Antimicrob. Agents Chemother. 53, 1670–1672.

Tsibris, A.M., Korber, B., Arnaout, R., Russ, C., Lo, C.C., et al., 2009. Quantitative deep sequencing reveals dynamic HIV-1 escape and large population shifts during CCR5 antagonist therapy in vivo. PLoS One 4, e5683.

Valarcher, J.F., Schelcher, F., Bourhy, H., 2000. Evolution of bovine respiratory syncytial virus. J. Virol. 74, 10714–10728.

Vignuzzi, M., Stone, J.K., Arnold, J.J., Cameron, C.E., Andino, R., 2006. Quasispecies diversity determines pathogenesis through cooperative interactions in a viral population. Nature 439, 344–348.

Vignuzzi, M., Wendt, E., Andino, R., 2008. Engineering attenuated virus vaccines by controlling replication fidelity. Nat. Med. 14, 154–161.

von Kleist, M., Metzner, P., Marquet, R., Schutte, C., 2012. HIV-1 polymerase inhibition by nucleoside analogs: cellular- and kinetic parameters of efficacy, susceptibility and resistance selection. PLoS Comput. Biol. 8, e1002359.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., et al., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

Weidt, G., Deppert, W., Utermohlen, O., Heukeshoven, J., Lehmann-Grube, F., 1995. Emergence of virus escape mutants after immunization with epitope vaccine. J. Virol. 69, 7147–7151.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., et al., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. 102, 9294–9299.