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Genetic Variability in RNA Viruses: Consequences in Epidemiology and in the Development of New Strategies for the Extinction of Infectivity

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15.1 Introduction

Viruses constitute one of the simplest biological entities in nature. They possess some properties typical of life, such as the transmission of genetic information through generations, but lack a proper metabolism and a system to translate the genetic information into proteins. This ambivalence places them at the border between living and non-living matter. To reproduce themselves, viruses are forced to infect a host cell, behaving as intra-cellular parasites. Despite their simplicity, viruses have been able to develop a wide repertoire of infection mechanisms and replication strategies to adapt to the broad diversity of the cellular world in order to execute their genetic program.

All known viruses consist of one or several genomic nucleic acid molecules, covered by protective layers. Usually, there is one protein capside that can be enclosed by a lipid bilayer membrane proceeding from the host cell. The number of proteins encoded by the viral genomes is rather small, making their success to replicate and give rise to an offspring dependent on their ability to take advantage of the enzymatic activities provided by the host cell. After infection, cellular protein synthesis is stopped and most of the subcellular machinery is directed to produce copies of the viral nucleic acids and proteins. These newly synthesized viral components are assembled inside the cell into mature virions that can infect other cells of the same organism or establish transmission chains between different individuals. The stability of these chains strongly conditions the survival of the virus in nature. Outside an adequate host, viruses can still persist for some time in a latent state in which they are unable to replicate and exposed to irreversible damage by physical conditions of the environment. When they interact with the specific cellular receptors of a suitable host, they can initiate an infection that, if successfully transmitted in the population, can seriously compromise the survival of the host species.

In all cellular organisms, the genetic information is contained in the DNA. Before being translated into proteins (the molecules that execute the functions

necessary for the performance of the cell), the DNA has to be copied to mRNA. But genetic information also needs to be maintained through generations, a process that takes place by means of DNA replication, in which many enzymatic activities are involved. In contrast to cells, viruses are more versatile and can use DNA or RNA to store the genetic information. DNA viruses can follow a scheme similar to that followed by cells to replicate their genomes and to synthesize their proteins. However, RNA viruses need another process, RNA replication, which is not among the functions carried out routinely in the cell [1]. Therefore, they have to encode and express the enzymatic activities necessary to copy their genomes. These enzymes are the RNA replicases and the reverse transcriptases, which in many cases are co-encapsidated with the nucleic acid during the assembly of the viral particles. In this way, they are available at the start of the infection.

All living systems must reach a compromise between the correct copy of the nucleotide sequence of their genomes and the ability to adapt to an environment that is continuously changing [2]. The generation of mutants upon replication provides the necessary diversity from which natural selection can choose the best adapted variants in a concrete environment. The observed divergences among mutation rates in different species suggest that possibly this character is selected depending on the variability of the environment [3]. Cellular systems are able to maintain a relative constancy in the intra-cytoplasmatic medium and because of that, they do not need a high genetic variability. Thus, evolution has emphasized the selection of a replicative machinery with several corrector activities that permit a high copying accuracy. However, even in the cellular world, mutation rate is not a fixed character that cannot be altered. It can be modified in response to environmental changes by selection of variants with higher or lower error rates. The isolation of hypermutator strains, which show deficiencies in some of the polymerase corrector activities, is frequent in conditions of environmental stress and is a proof of the versatility of mutability as a character that can be modified when the environment requires it [4, 5].

A relevant characteristic of RNA viruses is that they replicate their genomes with a copying fidelity several orders of magnitude lower than cellular DNA [6]. This fact has been interpreted as a consequence of the fluctuating environments that viruses have to face. High error prone replication, together with the short replication times and large population sizes typical of RNA viruses, instead of being a handicap for survival provides an extraordinary evolutionary advantage by permitting the generation of a wide reservoir of mutants with different phenotypic properties [7]. The high variability of RNA viruses facilitates their survival in presence of antibodies and other defence mechanisms produced by the immune system of the host. It also makes possible the acquisition of novel pathogenic properties that in occasions have allowed to cross species boundaries favouring the infection of alternative hosts [8, 9]. Finally, the heterogeneity of RNA virus populations makes it also difficult to eradicate diseases with antiviral drugs, due to the emergence of

drug-resistant mutants [10], a problem that will be treated in more detail in the next sections. Whether the high genetic variability of RNA viruses is a selected character, necessary for survival in high fluctuating environments, or it is simply a consequence of the lack of corrector activities of RNA replicases and reverse transcriptases is a debated question. However, the fact that DNA organisms, which usually live in constant environments, have evolved corrector activities, whereas RNA viruses have not, suggests that replication with high error rates is a selected character that strongly favours viral adaptation to fast changing conditions.

15.2 Replication of RNA Viruses and Generation of Genetic Variability

The first requisite for the evolution of any population is the generation of a significantly wide genetic diversity on which natural selection and genetic drift can act to shape the properties of the new populations generated at subsequent generations.

The genetic variation attained by RNA viruses is mainly the result of mutation and recombination, two processes that are dependent on the properties of the enzymes that replicate their genomes. Genome segment reassortment occurs during encapsidation and can add extra variability in the case of viruses with segmented genomes.

The replication of RNA viruses takes place through two main mechanisms that involve the use of different enzymatic activities [1]. Riboviruses, including many prokaryotic RNA viruses as well as many animal and plant viruses (poliovirus, influenza virus, hepatitis A and C viruses, etc.), replicate their genomes using RNA replicases that catalyze the RNA-dependent RNA synthesis. The template RNA can be of positive polarity (it can work as mRNA) or negative polarity (it is the complementary strand that is translated into proteins). Viruses with positive polarity genomes deliver the nucleic acid directly to the cellular ribosomes and begin infections with translation. In contrast, viruses with negative polarity genomes begin infections with transcription to obtain mRNA molecules that can be translated. Retroviruses, HIV-1 (human immunodeficiency virus type 1) being the best known example, replicate their genomes through a different mechanism with an intermediary step that consists in the copy of the genomic RNA to DNA. This process is catalyzed by the enzyme reverse transcriptase, an RNA-dependent DNA polymerase carried by the viral particle. The DNA obtained in this way is integrated in the host chromosome, being transcribed by the cellular enzyme RNA polymerase II to produce transcripts that can function either as precursors of mRNAs or as genomic RNAs that can be assembled into progeny viruses.

The lack of corrector activities of both classes of enzymes RNA replicases and reverse transcriptases results in high mutation rates, which have been estimated in 10^{-4} to 10^{-5} misincorporations per nucleotide copied. For a virus

with a genome length of 10,000 nucleotides, this amounts to the incorporation of one incorrect nucleotide per genome copied on the average [6,11]. Thus, each new viral genome differs from its parent at one or two nucleotide positions. The relative proportion of a specific mutant in the viral population depends on the rate at which the mutant is generated and on its fitness, which is defined as the ability to give rise to a progeny in competition with the rest of viruses replicating under certain environmental conditions [7]. The number of mutations that occur per time unit is also influenced by the number of replication rounds during that period, this is, the generation time. For viruses with similar error rate polymerases, the shorter the generation time, the larger the number of mutants that is produced in the same time interval.

Recombination takes place when a new genome is built from fragments belonging to different parental molecules. In RNA viruses, this process usually occurs by template switching during RNA or cDNA synthesis. Most studies suggest that recombination rates in RNA viruses are lower than in other organisms [12], although there are some notable exceptions, such as HIV-1 in which the recombination rate seems to be higher than the mutation rate [13]. Recombination can be a powerful mechanism to create advantageous genomes and to purge deleterious mutations in a very short time. However, the actual effects of recombination in RNA viruses have not been studied in detail, and it is not clear whether it is beneficial or it has a negative effect on fitness [14].

Genome segment reassortment occurs in viruses with segmented genomes and consists in the encapsidation in the same viral particle of genome segments proceeding from different parental viruses. Influenza viruses are the typical example in which this process has been responsible for antigenic shifts, probably resulting from combinations of segments of influenza virus of different specificity [15]. The natural reservoir of influenza is aquatic birds, although the virus can also infect domestic birds and mammals (human or pigs preferably). When a reassortant influenza virus emerges, its pathogenic potential can dramatically increase, because the infected host is not able to recognize the antigenic determinants of the new virus generated. These reassortant strains have been responsible for a number of pandemics through history and most studies suggest that a new influenza pandemic is unavoidable [16].

15.3 Structure of Viral Populations

The structure of viral populations results from the concerted action of the processes of mutation and selection acting in very large ensembles of replicating units. Population size fluctuations, which frequently take place during transmission of viruses in nature, constitute an additional and important factor influencing the extension of genetic diversity from which a new virus population will be generated.

The evolution and self-organization of heterogeneous populations composed by a large number of molecules subjected to error-prone replication and exposed to selection was first studied theoretically [17]. These studies

showed that, for large population sizes and after long growth times in a constant environment, a steady-state is reached where each mutant represents a constant fraction of the total population. This equilibrium population was called quasi-species [18, 19]. The most frequently occurring molecular species, usually the one with the highest fitness, is called the master sequence. This sequence is accompanied by a mutant spectrum, composed by an ensemble of variants that differ in one or several nucleotide positions that can be responsible for fitness variations in individual mutants. The number of nucleotide differences between two sequences is called the Hamming distance. The consensus sequence is defined as the sequence of the most represented nucleotides at each genomic position in the ensemble of genomes constituting the population. The correspondence between fitness values and sequences (or between phenotypes and genotypes) reveals that fitness landscapes (a surface in the genotype space representing the fitness of each genotype as a point placed at a different height) are rather rugged, since relatively small sequence differences can cause great differences in fitness values.

Analysis of RNA virus populations, either at the phenotypic or genotypic level showed that these populations have a structure similar to the molecular quasi-species described theoretically [20, 21]. They present a master sequence surrounded by a mutant cloud and, in the absence of a cloning method to separate individual genomes, only the consensus sequence can be determined. However, two main differences have to be taken into account when comparing theoretical and viral quasi-species. The first one is that viral quasi-species usually are not equilibrium populations because viruses are continually confronted with many environmental perturbations that cause variations in the fitness distribution of the population. The second difference is that viral fitness is not only determined by the genomic replicative ability. In spite of its simplicity, a virus must complete successfully many processes to originate an infective progeny. These include recognition of the cellular receptors, uncoating and release of the nucleic acid inside the cell, interaction with many enzymes and cellular structures, correct assembly to give rise to new viruses and exit out of the cell. The ability of a virus to perform correctly all these processes, together with the replicative ability of its genome, is what determines its fitness value. These differences introduce uncertainties and additional complexity to viral evolution compared to molecular evolution described theoretically.

15.4 Viral Quasi-Species and Adaptation

Viral quasi-species constitute very dynamical structures in which the processes of generation of new mutants, selection of the best adapted and elimination of the less fit are continuously acting. Quasi-species replicating during a long time in a near-constant environment in the absence of large population size fluctuations can present a low rate of fixation of mutations in the consensus sequence, despite the continuous occurrence of mutants that is characteristic of the underlying dynamics of the population. In this case, the quasi-species is

well adapted to the environment and can maintain a low rate of evolution, as determined by the stability of the consensus sequence. Many of the mutants generated are lethal or very deleterious and are eliminated or maintained at low frequency by the action of negative selection. In contrast, mutants with a selective advantage can be present at high frequency, even if they are produced at a low rate [20]. Neutral mutations are thought to be very restricted in RNA viruses because of their highly compact genomes [22]. Low rates of evolution have been described for viruses well adapted to their animal reservoir (the host in which the virus is usually maintained in nature) as influenza in birds or hantavirus in rodents. The same happens in the laboratory, where viruses are usually cultivated during years in the same cellular type. In both cases an almost invariant consensus sequence can be found, although the dynamics of the quasi-species is always dominated by the processes of mutation and selection acting in close concert.

The factors that promote the fixation of mutations in the consensus sequence are usually environmental changes that favour the selection of the best adapted genomes in the new conditions or drastic reductions in the number of individuals that will originate a new population, what is called population bottlenecks. The occurrence of genetic alterations with an adaptive advantage in the absence of environmental perturbations is also possible, although it happens more rarely. In this section, we will focus on some features that favour the action of positive selection and amplification of advantageous mutants. We will mention three examples that make enormously difficult virus eradication:

1. *Treatment with antiviral drugs.* When a viral infection is treated with an antiviral agent the usual outcome is that, after a short time of success, the treatment loses its efficacy. The failure is generally due to the presence in the mutant spectrum of some genomes able to resist the action of the drug. Usually, these genomes have lower fitness in the absence of the drug and they are maintained at low frequencies by the action of negative selection. The presence of the drug inhibits the replication of the sensitive genomes, but not of the resistant ones, which are selected and amplified. It can also occur that, at the beginning of the treatment, no drug-resistant genomes are present in the population. However, the high mutation rates and large population sizes of RNA viruses make highly probable that, after a variable time lag, a resistant mutant appears, which in a short time can dominate the population. Maybe, the most dramatic example of drug-induced resistance occurs in patients infected with HIV-1 in which variants resistant to all currently used drugs have been isolated [23, 24]. At present, the most effective treatment to control HIV-1 infection is the so called highly active anti-retroviral therapy (HAART), which involves a combination of several drugs, aimed at preventing the emergence of variants with mutations conferring resistance to all the drugs at the same time.

2. *Antigenic drift.* There are considerable differences in the nature and duration of the immune response elicited by different viruses [25]. Some human viruses, such as measles or chicken pox, can only infect once, because their antigenic determinants have very slow evolution rate and the immunological response of the memory cells continues being effective along the whole life of the individual. In contrast, there are other viruses, influenza being the paradigmatic example, that can infect the same organism repeated times. Most experimental evidence leads to the conclusion that the tolerance of a virus to accept immune-escape mutations is limited by the restriction of conserving the cell tropism [26]. Modifications in the capside antigen domains of measles virus seem to have very deleterious effects, possibly because they affect the recognition of the cellular receptors. In contrast, influenza can experience a continuous change in the antigenic properties of the two main surface proteins involved in the entry of the virus inside the cell, the hemagglutinin and the neuraminidase. The evolution of the virus seems to be strongly influenced by selection of new antigenic variants to escape the immune system at the same time that the capacity of interaction with the cellular receptor is preserved [15]. In the case of the hemagglutinin gene, 18 codon sites have been identified in which non-synonymous nucleotide substitutions are much more frequent than synonymous [27, 28]. The remaining sites show the more common pattern of synonymous substitutions, indicating that possibly they are subjected to stronger evolutionary constraints.
3. *Change of tropism.* Many viruses are maintained in nature in animal reservoirs that do not manifest symptoms of disease. This probably occurs because the relation virus–host is very old (hundreds or thousands of years) and both species have had enough time to co-evolve, meaning that the virus has attenuated its virulence and the host also has acquired some properties that permit coexistence with the pathogen. The long time of evolution in the same host has permitted to these viruses to be close to the equilibrium between mutation and selection processes and to maintain a high stability in the consensus sequence. Occasionally, a virus well-adapted for replication in a particular host can cross the species boundaries and infect a new host. This can be facilitated by genetic changes in the virus and/or by ecological factors that involve alterations in the relationships established among different species in nature [29, 30]. The infection of a new host constitutes a sudden change in the environment in which viral replication takes place, usually with the consequence of a drastic decrease in the average fitness of the virus population, which prevents further transmission. The success of a virus to establish as a new infectious agent in the new host relies largely on two features (a) its ability to interact with a cellular receptor that permits the entry inside the cells and (b) the acquisition and fixation of mutations that allow efficient replication and capacity of transmission between organisms. Most recent virus emergences in humans include HIV-1, whose closest animal ancestor seems

to be the simian immunodeficiency virus found in a particular species of chimpanzees (SIVcpz) [31], the coronavirus causing SARS (severe acute respiratory syndrome) [32] and the influenza virus H5N1, an avian virus strain that can infect directly humans without further human-to-human transmission [33].

15.5 Population Dynamics of Host–Pathogen Interactions

Virus dynamics in nature cannot be separated from host population dynamics, constituting two processes in continuous interaction [25, 34, 35]. Factors such as the transmission mode, the basic reproductive number (R_0), the duration of the infectious period, the renewal of susceptible hosts and the durability of the immune response contribute to shape the genetic heterogeneity of viruses and the quasi-species structure. They also strongly condition the evolution of the pathogen along the time, adding a great complexity to the epidemiological and phylogenetic studies on RNA viruses.

An important factor in viral evolution that takes place at the inter-host level is the number of viral particles that are transmitted from one host to the other [20]. When this number is very small, a population bottleneck takes place. Then, only one or few individuals originate a new population, resulting in a strong reduction in the genetic diversity. The consequence is that any mutation present in the founder genomes will have a high probability of being transmitted to the progeny, accelerating in this way the rate of fixation of mutations (Fig. 15.1). Since most mutations are deleterious, the expected effect of their accumulation through repeated bottlenecks is a decrease in the

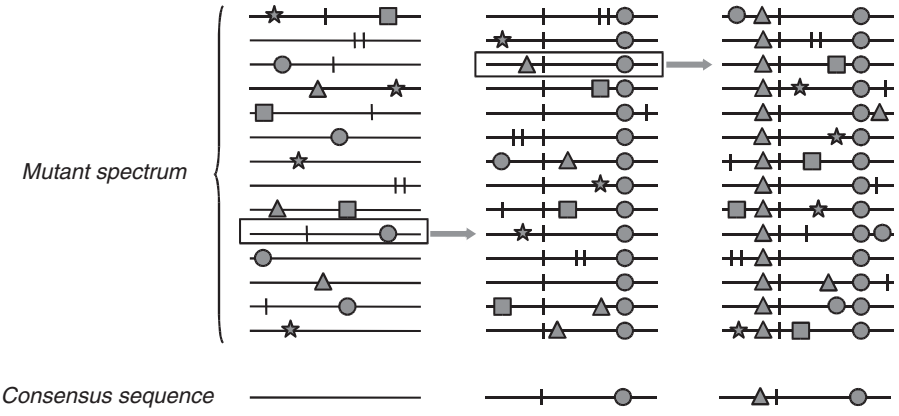

Fig. 15.1. Accumulation of mutations in the consensus sequence of a heterogeneous virus population when a single genome (*in the box*) is selected to found a new population. All the mutations carried by this genome are transmitted to the progeny, and consequently they will be fixed in the consensus sequence

average fitness that eventually could lead to the extinction of the population. Bottlenecks are very frequent in nature, during the inter-organ or inter-host transmission of many viruses. Thus, in each new infected organism, the quasispecies must be rebuilt from one or a few founder genomes, a fact that could lead to a wide diversity in diseases in which the usual form of transmission is mediated through bottlenecks. The persistence of viruses in nature and the limited number of circulating strains in diseases such as influenza, despite the frequent occurrence of bottlenecks, is paradoxical [36]. It is believed that the inter-host competition that can induce stochastic losses of the less fit variants, together with the action of previous immune responses on genetically related virus variants (the so called cross-immunity) are factors that restrict the strain diversity. The intra-host competition that takes place after each transmission event is an additional factor that favours the optimization of the viral population inside each infected individual and also contributing to the resistance to extinction of viruses transmitted through bottlenecks.

Grenfell et al. [37] have classified RNA viruses in four phylodynamic categories, according to factors pertaining to the host–pathogen interactions (mainly the duration of the infection and the nature and strength of the immune response). They are briefly described:

1. *Short infections with strong cross-immunity.* The best known viruses included in this category belong to the family of morbilliviruses (measles being a well studied example). In these viruses, epidemic cycles are mainly determined by the lifelong immunity elicited by the pathogen, which causes that the renewal of susceptible hosts takes place only at the birth of new individuals. The existence of a strong immune response that is powerful against all circulating strains (strain-transcending immunity) would prevent the action of selection. In these viruses, the burden of many different strains seems to be limited by spatio-temporal parameters of the dynamics of the epidemic process.
2. *Short infections with partial cross-immunity.* The best example of this category is influenza A virus. The high mutation rate characteristic of RNA viruses, together with the transmission of influenza through bottleneck events, opposes to the limited variability within lineages. In contrast to measles, cross-immunity against virus variants is only partial and the replacement of susceptible individuals takes place, not only through the birth of new hosts but also through generation of new influenza strains that may affect individuals previously exposed to the virus. Evolution of influenza and its epidemic dynamics have been modelled in several studies, trying to reproduce the strong seasonality of infections and the replacement of strains at each epidemic. The most successful models reproduce the behaviour of influenza epidemics when a short-lived strain-transcending immunity (in contrast to the long-lived immunity characteristic of viruses in the previous category) is included as an essential factor limiting viral diversity in the host population [36]. However, the role

of within-host dynamics after each bottleneck mediating transmission remains to be added to the epidemic model.

3. *Infections with immune enhancement.* These are infections with the possibility of antibody-dependent enhancement (ADE). An example is dengue virus that comprises four serotypes co-circulating in tropical regions. ADE causes that secondary infections produced by a different virus serotype usually curse with more severe symptoms than primary infections.
4. *Persistent infections.* In this category are included viruses such as HIV and HCV (hepatitis C virus) that can persist in their host during long times periods. For these viruses, inter-host dynamics is slow, being more important and faster the intra-host period of evolution that is driven by continuous and strong immune pressure.

15.6 The Limit of the Error Rate

Since the high genetic heterogeneity of RNA viruses provides an enormous adaptive capacity, it could be naively expected that additional increases in the replication error rate makes evolutionary adaptation even more efficient. However, there are many theoretical and experimental evidences showing that RNA viruses have selected the maximal error rate, which is compatible with the preservation of their genetic information.

Theoretical studies on molecular evolution postulate that the higher the error rate and the genome length, the smaller is the probability of obtaining a progeny identical to the parental genome and to conserve the master sequence in the population [17,19]. There is a sharp limit, called error threshold, which cannot be crossed without catastrophic consequences for the survival of the population (see the chapter by Jain and Krug in this book). Below this limit, the quasi-species can maintain a large genetic variability from which the best adapted molecules are selected. When the threshold is crossed, the dispersing force of mutation cannot be compensated by selection of the best adapted phenotypes and the genetic information melts away in a process with the physical characteristics of a first order phase transition, as the melting of a solid. The transition takes place in an 'information space' that is multidimensional, comprising 4^N sequences of length N [38].

The error rate that can be maintained is related to the genome length according to this relation:

$$N_{\max} < \ln s_0/(1-q)\,. \tag{15.1}$$

Here $N_{\max}$ is the maximal length of the genome and it is inversely proportional to the error rate per nucleotide $(1-q)$. The factor s_0 indicates the selective advantage of the master sequence in relation to the mutant spectrum. Measurements of the chain lengths and the replication error rates of RNA viruses show that the genome lengths of RNA viruses are close to the maximum that can be maintained at the error rates of their replication. Moreover,

phylogenetic analysis of RNA viruses reveals a negative correlation between rates of nucleotide substitution and genome size [39]. As a direct consequence, all viral functions must be encoded within a limited genomic space (10–15 kb on average for most RNA viruses), meaning that certain regions of the genome will often have to participate in several functions at the same time, resulting in restrictions to the capacity of RNA virus to alter their nucleotide sequences. The most frequent evolutionary constraints identified are the following [22]:

1. Usually, the antigenic determinants of a virus are domains of the same proteins involved in the recognition of the cellular receptor [26]. This fact restricts the possibilities of immune escape to the occurrence of mutations in domains that are not crucial for penetration of the virus inside the cell.
2. Genomic coding regions can also be involved in the interaction with enzymes or cellular structures and in the regulation of the correct synthesis and assembly of the viral components to constitute mature particles.
3. Synonymous mutations may be not silent and have effect on fitness because they can affect the secondary structure of RNA domains critical for keeping the stability and functionality of the molecule [40].
4. Sometimes the same genomic region can encode several proteins through the use of overlapping reading frames.

In most RNA viruses, a high amount of particles is not infectious, suggesting that viral populations operate near the error threshold and most mutations are not easily tolerated, possibly due to the above-mentioned constraints. The large population sizes constituted by RNA viruses seem to be necessary to avoid stochastic extinctions that could happen due to the generation of many deleterious mutants.

Given the high mutation rate of RNA viruses, and the increased fraction of deleterious mutations over advantageous ones that occur when a population is well adapted to the environment, one can think of two alternative strategies for driving a viral population to extinction. Both of them involve an increase in the number of mutations in individual viral genomes, which can be related, although not necessarily, to changes in the consensus sequence of the population.

The first pathway is the classical one described by molecular evolution error catastrophe theories. It consists in the increase of the replication error rate, usually through the use of mutagens. The new populations generated exhibit larger complexity than the initial ones. Advantageous mutations, even if they occur, would be spoiled by the continuous generation of deleterious mutations, before they can be fixed by natural selection. In this case, it is the strong dispersing force of mutation what dominates the dynamics of the population.

The second pathway consists in the application of successive bottlenecks to the population. After each bottleneck, the founder genomes give rise to a new population through a limited number of replication rounds. The larger the number of generations between bottlenecks, the closer is the new population to

the equilibrium between mutation and selection [41]. The resulting populations have two essential characteristics. The first one is their low complexity, because the low number of copy rounds taking place between bottlenecks does not permit to generate a large genetic diversity. The second one is an increased rate in the fixation of mutations in the consensus sequence, since most mutations present in the founder genomes are transmitted to the descendants (Fig. 15.1). Given the high amount of deleterious mutations, the expected result of their accumulation is a progressive reduction in the average fitness of the population that could lead to the extinction of infectivity.

The structure of the viral populations generated through the two pathways described here have different evolutionary consequences that have been explored experimentally by several groups. Next sections contain a review of the main results published in this field.

15.6.1 Increases in the Error Rate of Replication. Lethal Mutagenesis As a New Antiviral Strategy

There are many experimental evidences documenting extinction of RNA viruses experiencing an increased mutation rate due to the action of mutagens [42–47]. The mutagens most currently used are 5-fluorouracil (FU), 5-azacytidine (AZC), azidothymidine (AZT), ribavirin and 7-hydroxyurea. Some of them are nucleoside analogues that, in addition to increasing the rate of erroneous incorporation of nucleotides, can also interfere with other cellular or viral processes, such as endogenous nucleotide metabolism, viral replication or transcription.

Foot-and-mouth disease virus (FMDV), poliovirus, HIV-1 and lymphocytic choriomeningitis virus (LCMV) are some examples of RNA viruses in which successful extinctions of infectivity have been documented. The results agree with molecular evolution theories that postulate that viral replication operates very close to an error threshold that cannot be crossed without compromising the transmission of genetic information and the existence of the population (reviewed in [48]). Although many studies have been devoted to the characterization of the mutant spectrum of pre-extinction populations [43, 49, 50], it is not clear how the quasi-species looses its infective capacity. It is not known whether all the genomes are carrying lethal mutations and therefore are unable to replicate or it is the disorganization of the mutant spectrum what makes the quasi-species to be non-infective. In the last case, the quasi-species could still conserve some viable genomes that, in the absence of the interfering mutants, could initiate the development of an infective population.

Mutagenized populations of FMDV treated with AZC and FU could be efficiently extinguished [47]. As expected, the characterization of the RNA genomes composing the pre-extinction populations did not show mutations in the consensus sequences, but displayed an increase in the complexity of the mutant spectrum (reviewed in [51]). The maximum increases in complexity occurred in the polymerase gene, which usually is well conserved. Other studies

have also demonstrated the invariance of the consensus sequence, despite the occurrence of a high number of mutations in individual genomes [43].

The same mutagenic agent can behave differently in different viruses. As an example, LCMV was systematically extinguished after only two or three passages in the presence of FU [43, 52], whereas extinction of FMDV was stochastic and required a larger number of passages in the presence of similar amounts of mutagenic agent. Differences in the susceptibility of a virus to a mutagen can be explained by a number of factors including different affinity of the polymerase for the mutagen, effect of the mutagen in other viral or cellular processes, type of mutations preferentially induced by the mutagen that can affect viral functions differently depending on the nucleotide composition of the virus genomes, etc., [53]. The influence of variations in the mutation rate of different virus polymerases in the capacity of mutagens to extinguish infections is not well known. In principle, it should be expected that the closer is the virus to the error threshold, the easier should be its extinction by increased mutagenesis. However, the error rate of the polymerase is very difficult to estimate, and it can change depending on environmental factors and the region of the genome sequenced. Mutation rates are usually obtained from measurements of mutation frequency, a procedure that can lead to underestimation of the true mutation rates, because only replicating genomes are abundant enough to be detected. The isolation of a poliovirus mutant with a high fidelity polymerase [54, 55] that is resistant to the action of ribavirin and other mutagens clearly indicates that the error rate of a particular polymerase is a relevant factor contributing to the efficiency of increased mutagenesis to extinguish viral infections.

Studies in both riboviruses and retroviruses suggest that host enzymes also represent a potential source of variation by RNA editing [56]. There are some cellular enzymes able to produce hypermutation in the viral genomes, which occurs as clusters of specific base substitutions. A documented example is the enzyme APOBEC3G, which has been shown to generate G→A hypermutations in HIV-1. Enzymes of this type could act as a natural strategy for limiting viral infection by increasing mutagenesis above the error threshold. The discovery of these host factors constitutes an alternative for the development of agents that specifically enhance the natural antiviral activity of cells.

Recently, extinction by lethal mutagenesis has been shown to involve more complex mechanisms than those affecting only the replicative ability of genomes [44]. It is well known that in a normal infection a variable amount of the viruses produced are non-infective because they are unable to code for all functional proteins [57]. However, inside the cell, it is plausible that many of these non-infective genomes behave as parasites and replicate using the proteins produced by other viruses. When the mutation rate is kept below a critical threshold, defective mutants maintain an equilibrium with viable genomes. The increase in the mutation rate forces the appearance of a larger amount of defective genomes that, beyond a critical fraction, can exhaust the

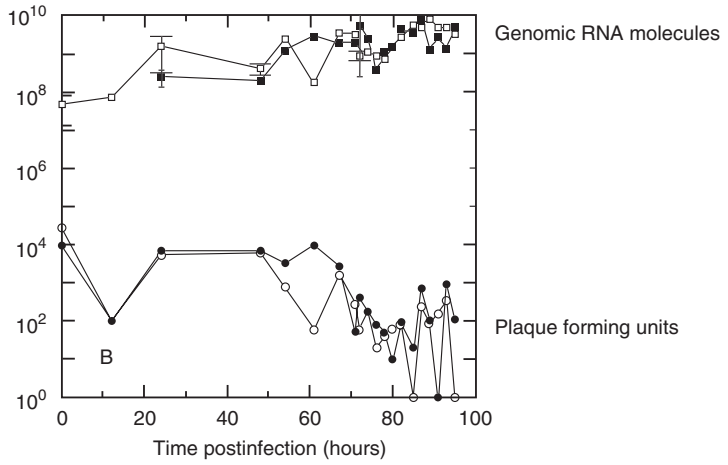

Fig. 15.2. Quantification of the viral genomic RNA and infectivity in supernatants and cell fractions of LCMV incubated with 100 $\mu g\,ml^{-1}$ of 5-FU. Although a high amount of RNA is still present in the samples, infectivity declines until undetectable levels, indicating that replicative ability does not disappear simultaneously with infectivity. Further details of this experiment can be found in [44]. *Open symbols* correspond to the intra-cellular fraction. *Filled symbols* correspond to the supernatant fraction

resources necessary for viral replication, becoming an additional force that can promote extinction.

This conceptual framework derives from several 'in vitro' experiments with LCMV [44]. Infective viruses and RNA genomic molecules were monitored during a virological steady-state persistent infection of BHK-21 cells by LCMV in the absence and presence of 5-FU (Fig. 15.2). In the course of the infection, there is a clear increase in the number of genomic RNA molecules, both in the intra-cellular fraction and in supernatants of control and mutagenized virus. However, in FU-treated virus cultures, infectivity declines and falls below detection, despite the high number of genomic RNA molecules. The number of infective units per RNA molecule as a function of the mutation frequency yields a curve with a sharp decay when the mutation frequency overcomes a critical threshold. The sudden loss of infectivity takes place through a transition analogous to that predicted by error catastrophe theories. However, the unexpected outcome of the experiment was the presence of large numbers of RNA molecules, revealing that the replicative ability does not disappear simultaneously with infectivity. Similar results have been found with poliovirus and Hantaan virus where decreases in infectivity preceded decreases in viral RNA levels [42, 58, 59].

Lethal mutagenesis probably presents many of the same difficulties as conventional antiviral therapy. An important problem takes place in viruses, such as retroviruses, that can stay in a latent state during a long time in cellular

or anatomical reservoirs. Activation of these latent viruses can contribute to the resurgence of the disease after interruption of drug treatment in vivo [60]. However, the strongest obstacle to antiviral mutagenesis is the appearance of drug-resistant mutants due to the presence of enhanced fidelity polymerases. Possibly these mutants are less able to generate resistances to other antiviral drugs, due to the diminished ability of adaptation that results from the reduction of the genetic diversity because of the higher fidelity of the polymerase. Therefore, combined therapies consisting of lethal mutagenesis and other antivirals could be a promising strategy for the treatment of viral infections [54].

15.6.2 Evolution of Viral Populations Through Successive Bottlenecks

The probability of extinction of small asexual populations due to the accumulation of mutations was first studied by Muller several decades ago [61]. He predicted that the genomes with the lowest mutational load could be stochastically lost due to population fluctuations through a mechanism similar to the clicks of a ratchet. When the ratchet clicks the first time, this means that the genomes with no mutations are lost and the least loaded class corresponds to individuals carrying one mutation. In the next click, the one-mutation class disappears by a similar mechanism, and the least mutated class corresponds now to genomes with two mutations and so on. At that time it was believed that the least mutated genomes were the best adapted and that reversions were the only mechanism able to recover fitness. Thus, this process, which is particularly effective at high mutation rates, as it happens in RNA viruses, should inevitably imply a progressive fitness loss that can lead populations to extinction.

The experimental study on the transmission of RNA viruses through successive bottlenecks usually is carried out making serial plaque-to-plaque transfers (Fig. 15.3). At each transfer, the viral population is plated at low multiplicity of infection to get well-isolated lytic plaques that are the result of the infection by a single virus, which after several replication rounds gives rise to a progeny. Since at each transfer the effective population size is reduced to one individual, this constitutes the most extreme form of bottleneck. The population contained in a randomly chosen plaque is isolated, properly diluted and plated again in a process that is serially repeated. The consequences on fitness of successive repetitions of this process have been analyzed with several RNA viruses including bacteriophages MS2 [62] and Phi 6 [63], vesicular estomatitis virus (VSV) [64–66], FMDV [67–69] and HIV-1 [70]. In all these studies, progressive fitness declines were found, although extinctions of infectivity were only observed in the case of HIV-1.

The most complete study on the effect that the accumulation of mutations through plaque-to-plaque transfers has on fitness evolution has been carried out with FMDV [67–69, 71].

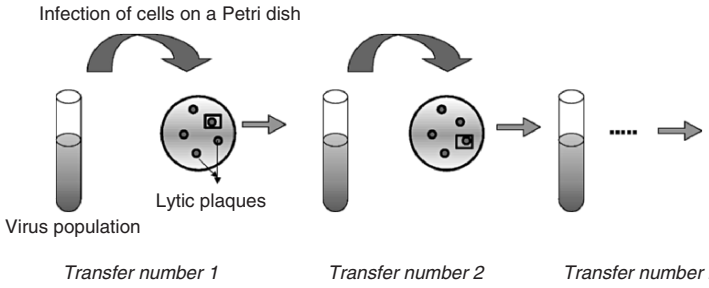

Fig. 15.3. Schematic representation of the experimental procedure of plaque-to-plaque transfers. The starting viral population is plated to isolate individual lytic plaques. The virus contained in a single plaque (in the box) is diluted for titration of infectious particles and to be used in the successive plaque transfer. The process is repeated as many times as desired

In this study, the titer of the plaques (determined as the number of infectious units per plaque or pfu) at each transfer was taken as a measure of fitness. The mutations that accumulated along the process were identified by determining the consensus sequence of the viral population isolated from single plaques at different transfers. The expected result of the experiment was a progressive decrease in fitness accompanied by an increase in the number of mutations fixed in the consensus sequence. After a certain number of transfers, extinctions of infectivity were expected. In contrast to these expectations, a biphasic dynamics of fitness decrease was observed. There was an initial period of roughly exponential fitness loss, but after a variable number of passages, a statistically stationary state of fitness with large fluctuations around a mean constant value was reached (Fig. 15.4). In this state, the virus exhibits a great resistance to extinction, since when it reaches a very low fitness value, the usual outcome at the next passage is a sudden fitness recovery. A detailed statistical analysis of the viral titers at the stationary state showed that fluctuations in the viral yield followed a Weibull distribution [69]. This distribution is indicative of an underlying dynamics with two main features (a) an exponential amplification of the founder genomes during the development of each plaque, which makes that small fitness differences are considerably amplified and (b) large variations in the initial state of the system at each transfer, which is determined by the stochastic nature of the sampling process.

Strikingly, mutations accumulated at the same rate in the phase of fitness decrease and in the stationary state [68]. This might indicate that the nature and effects of mutations can vary with the transfer number, depending on the restrictions imposed by the selection of the genomes able to form plaques. When the population is well adapted to the environment, as it happens at the beginning of the experiment, deleterious mutations are well tolerated. However, as the population is getting more debilitated, less deleterious mutations can be accepted and possibly there are many extinctions of individual genomes

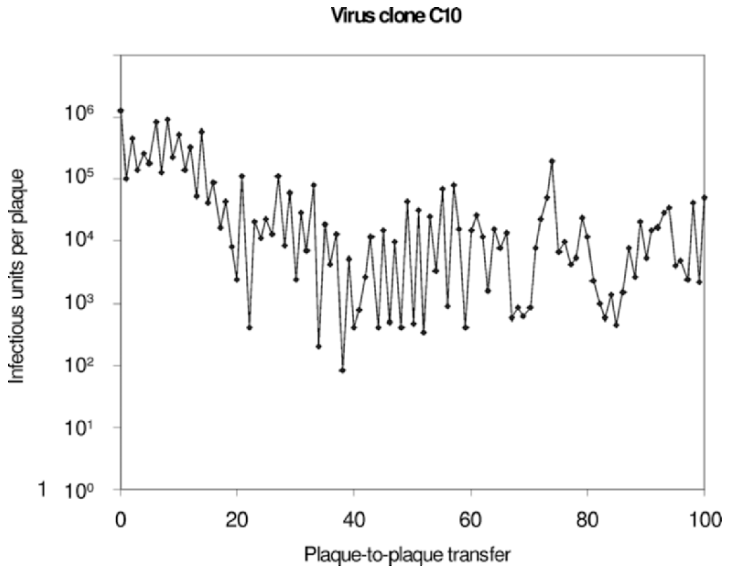

Fig. 15.4. Infectious units per plaque produced along the process of plaque-to-plaque transfers experienced by the viral clone C10. After an exponential decay of infectivity, a statistical stationary state with strong fluctuations is attained

that become unable to replicate. Nevertheless, a fraction of the genomes contained in a plaque can possess advantageous mutations, in some occasions because the mutation has a positive effect 'per se' and in others because it has a compensatory effect in a concrete genome carrying a particular combination of mutations. In the stationary state, where average fitness values possibly are the lowest ones compatible with virus survival, advantageous mutations would be more easily selected, because only the genomes carrying them can form plaques and be chosen for the next transfer. Each advantageous mutation produces a fitness increase that moves the genome to a different position in the fitness landscape. This permits the acceptance of additional deleterious mutations, originating the fluctuating pattern of infectivity that is observed in the experiments.

An interesting result is the preferential accumulation of mutations in certain genomic regions that present a mutation frequency significantly higher than the average obtained considering the whole genome [68] (Fig. 15.5). An unusual distribution of mutations has also been found in bottlenecked HIV-1 clones in which there was a higher accumulation of mutations in the gene gag and the first third of the genome, compared to the gene env, which is less conserved in natural populations of the virus [73]. Bottlenecked VSV clones also accumulated a high number of mutations in the N open reading frame, contrasting with the conservation of this region in natural isolates [66]. All these results suggest that bottlenecks permit the isolation of genomes that

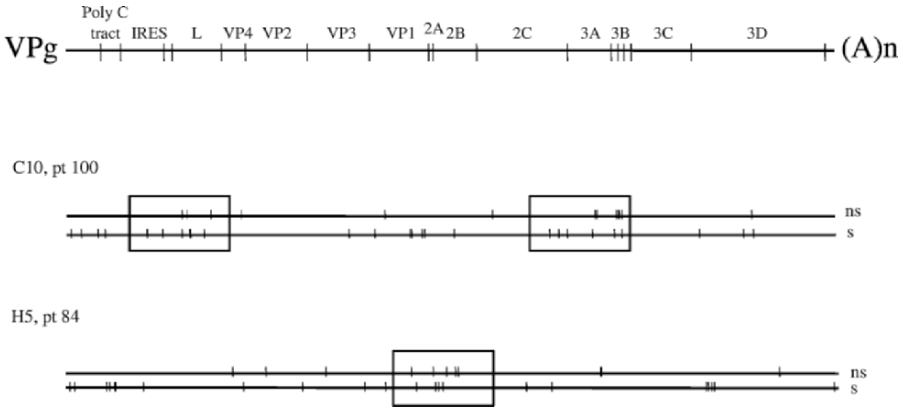

Fig. 15.5. Location of the mutations found in the genome of FMDV clones C10 and H5 subjected to 100 and 84 plaque transfers respectively. The *top horizontal line* is a scheme of the FMDV genome showing the main regulatory regions and the encoded proteins [72]. The *lines* below the genome indicate the non-synonymous (ns) and synonymous (s) mutations present in the virus. The *boxes* indicate the genomic regions where the number of mutations is significantly higher than the average for the whole genome

otherwise would be eliminated under the action of positive selection that dominates virus optimization. Genomic regions that seem to be much conserved might mutate with the same mutation rate as the rest of the genome, although subjected to stronger constraints. Nevertheless, the evolutionary relevance and the molecular mechanism by which the mutation clusters observed in the bottlenecked FMDV clones are generated is unknown and further experiments are in progress to answer this question.

A numerical model of evolution through bottlenecks was developed with the aim of identifying the parameters that are responsible for the biphasic dynamics of fitness loss [74, 75]. The main features of the model are the occurrence with low probability of advantageous mutations and the presence of an extinction threshold, which means that genomes reaching the minimal allowed fitness value are eliminated. The results of the simulations were very similar to those observed in the experiments: a biphasic dynamics of fitness decrease and large fluctuations in the fitness values attained at the stationary state. Moreover, the statistical analysis of fitness values reveals that, similarly to the experimental results, they follow a Weibull distribution, strongly supporting that the underlying dynamics must be the same in both the simulations and the experiments. The elimination of individuals as their fitness falls below the extinction threshold and the probability of selecting for the subsequent transfer genomes with compensatory mutations constitute two factors acting in close concert to avoid extinctions due to an excessive accumulation of deleterious mutations. The occurrence of compensatory, advantageous mutations was not introduced in most models of Muller's ratchet that considered that back

mutations were the only mechanism to revert the negative effect of deleterious mutations [76,77]. However, compensatory mutations are much more frequent than reversions as a mechanism to increase fitness, as it has been demonstrated in several theoretical and experimental studies [78, 79]. Accordingly, during the process of fitness recovery of FMDV and VSV bottlenecked clones upon large population passages, both reversions and compensatory mutations were found to be responsible for the observed fitness increases [64,66,71]. None of the recovered strains reverted to a wild type sequence, confirming that bottlenecks move the quasi-species through the fitness landscape towards regions where the adaptive value of mutations can be drastically altered.

The results of all these studies show that there are different mechanisms able to modulate the adaptive value of mutations. When the environment is altered, a new fitness landscape appears where the effect of particular mutations varies. In a similar way, even if the fitness landscape is not modified, bottlenecks constitute an effective way to explore new regions, where the selective value of mutations can differ from that present in the initial quasi-species. This means that the effect of mutations can vary depending on the mutations previously accumulated in the genome, a fact that points to epistatic interactions. Sanjuán et al. have studied the effect of pair of mutations in the VSV genome, compared to their effects as single mutations [80]. They found mainly antagonistic interactions between deleterious mutations (the effect of both mutations appearing together is smaller than the sum of the separate effect of each mutation). This finding can partially explain the non-linear dynamics of fitness loss observed in the FMDV clones. Some theoretical studies also show that antagonistic epistasis can reduce the speed of the ratchet.

A relevant question concerns the effect that the high mutational load of viral populations with a long history of bottlenecks has on their adaptability. The studies of Novella [81] have shown that bottlenecked viruses, even if they have recovered fitness through massive passages, always loss in competition experiments with the wild type, meaning that they have lower adaptability. It would be quite interesting to investigate if the high number of mutations accumulated in bottlenecked viruses also has negative consequences for adaptation to a new environment with a different fitness landscape. These studies can be carried out with viruses carrying different combinations of mutations and having the same fitness value, as those obtained at different transfer number in the stationary state attained by FMDV bottlenecked clones. The results of experiments of this type would allow to get more insight in the alternative adaptive solutions that can be explored by RNA virus populations differing in the consensus sequence.

15.7 Conclusions

Most of the viruses that are important human pathogens have RNA as genetic material. All of them share high mutability and a great potential for adaptation that makes their eradication enormously difficult. The isolation of

drug-resistant mutants, the emergence of new diseases in humans caused by viruses that usually are maintained in animal reservoirs or the appearance of viral variants able to resist the action of the immune system of the host constitute important challenges for research in this century. One of the most promising strategies for the control of viral diseases consists in the increase of the error rate of viral replication above the threshold that prevents further transmission of genetic information. The difficulty to apply lethal mutagenesis to the treatment of viral infections largely rely, as it happens with other antiviral drugs, on the emergence of resistant mutants, which in this case would probably be those carrying high fidelity polymerases. The knowledge of the exact mechanisms leading a population to error catastrophe implies a detailed study of the composition and structure of the mutant spectrum of the quasi-species. In this sense, the comparison with the structure of bottlenecked populations that have accumulated a large number of mutations still compatible with survival can help to design new strategies for the extinction of infectivity.

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