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Minority Report: Hidden Memory Genomes in HIV-1 Quasispecies and Possible Clinical Implications

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Abstract

The RNA viruses replicate as complex distributions of closely related genomes termed viral quasispecies. The behavior of the evolving quasispecies and its response to selective pressures such as antiviral treatment is influenced by the ensemble of mutants that compose the viral population. One such influence is the presence of minority subpopulations in the mutant spectra of viral quasispecies. Biologically relevant mutants have long been known to be present as minority components of replicating viral populations. However, experiments designed with specific mutants of the animal pathogen foot-and-mouth disease virus in cell culture explained the presence of a class of minority genomes termed memory genomes. They descend from those variants that were dominant at an earlier phase of quasispecies evolution, and arise as a consequence of quasispecies dynamics, when viral populations are subjected to discontinuous selective pressures. The presence of memory genomes has also been documented during intrahost evolution of HIV-1 in vivo***. The analysis of sequential viral samples of different HIV-1-infected patients showed that two distinct types of memory can operate in retroviruses: a replicative memory analogous to that observed in foot-and-mouth disease virus, as well as a reservoir memory derived from the integrative phase of the retroviral lifecycle. Despite being hidden as minority components of the HIV-1 viral population (ranging from about 0.1 to 20% of the total number of genomes in the quasispecies analyzed), memory genomes can drive the evolution of the virus during HIV-1 infections under antiviral therapy. The limited availability of current experimental data on minority HIV-1 subpopulations*** in vivo ***implies that further studies are required in order to define the cutoffs of clinically relevant minority genomes. Nevertheless, it is already evident that such low-abundance genomes remain undetectable by traditional genotyping methods such as consensus sequencing or conventional hybridization techniques. Several experimental systems are currently available for the detection and characterization of minority components of the mutant spectra of viral quasispecies including HIV, hepatitis C virus and hepatitis B virus. Some of these biotechnological approaches could, in the near future, be taken over and exploited in the clinical setting as useful biosensors with which to improve the management of HIV-infected patients.*** **(AIDS Rev. 2008;10:93-109)**

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Key words

Quasispecies memory. Intrahost evolution. Reemergence. Persisting minority variants. Drug resistance. Pyrosequencing. DNA microarrays.

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Introduction

Viruses with an RNA genome are the most abundant group of human, animal, and plant pathogens. Their replication is characterized by very high mutation rates, with an average of 10^{-4} substitutions per nucleotide

copied, due to absent or low proofreading activity in their DNA-dependent RNA polymerases (reverse transcriptases, in retroviruses including HIV), or their RNA-dependent RNA polymerases (in the case of riboviruses such as hepatitis C virus)[1,2]. As a consequence, RNA viruses evolve as highly heterogeneous populations that contain particles with closely related but non-identical genomes. Such a complex population structure is termed "viral quasispecies"[3-6]. Quasispecies dynamics is characterized by a continuous process of mutant generation, competition and selection, resulting in the dominance of one or several most-fit genomes surrounded by a mutant spectrum[7], as depicted in figure 1. Quasispecies structure and large population sizes allow RNA viruses to react quickly to selective pressures exerted either by the immune system of the host, or by the administration of antiviral drugs or monoclonal antibodies (MAb)[8,9].

Several decades of experimental research on different RNA virus families, as well as the development of detailed mathematical models, have documented that the target of evolutionary selection is the whole viral population rather than individual viral particles[4,5,7,9-12]. Indeed, recent computer simulations of molecular quasispecies dynamics have shown that the adaptive behavior and fate of evolving quasispecies cannot be explained by the features of any particular genome in the mutant spectrum, not even those of the most abundant one. Quasispecies behave as the result of a collective search through the sequence space of the population as a whole, whose properties are distributed and delocalized among the mutants that compose the quasispecies[13]. Therefore, the evolution of viral quasispecies, such as HIV, hepatitis C virus (HCV), hepatitis B virus (HBV), influenza viruses, and other important human pathogens, as well as their response to selective pressures exerted by antiviral treatment is influenced by the complexity of the ensemble of mutants that compose the evolving population. As a consequence, the investigation of the effects that the internal structure of the quasispecies produces on the pathogenesis is becoming an issue of major interest in virology[6,12,14-16].

In particular, it is currently recognized that although traditionally ignored, minority genomes present in the mutant spectra of viral quasispecies may play a biological role with relevant consequences in the clinical setting. The reported clinical influences of low-abundance subpopulations within viral quasispecies include the dynamics of reappearance of founder viruses during the natural history of several RNA virus infections[17-23], the shifts in HIV-1 coreceptor tropism at different phases of the infection[24], the evolution of HIV-1 antiviral drug resistance[25-39] and the reemergence of HIV-1 genomes after treatment interruptions[40-45]. A relevant example of the key role of minority HIV-1 genomes in the further clinical evolution has been recently underlined[46,47]. These studies have documented the lack of sensitivity to nevirapine (NVP) and other nonnucleoside reverse transcriptase inhibitors (NNRTI) in pregnant women whose HIV-1 quasispecies harbored minority subpopulations (undetectable by conventional methods) of NNRTI-resistant genomes. In certain cases the effect of such low abundance, hidden viruses was magnified by the fact that the single dose NVP given at the time of delivery could have been inactive and therefore unable to prevent vertical transmission of HIV.

Here we review some biological consequences of minority genomes and the main methods available to detect them, with emphasis on our own work on a class of minority genomes termed "memory genomes". The initial experiments involved measurements of the frequencies of specific mutants of foot-and-mouth disease virus (FMDV) that after gaining dominance in the population, decreased in frequency to levels that could not be accounted for by mere mutational pressure on the parental genome. Such history-dependent minority genomes reflected those that were dominant at an earlier evolutionary phase of the same lineage. For this reason, they were called memory genomes[48,49]. Memory genomes were also identified in HIV-1 *in vivo*[50,51] and, although undetectable by conventional genotypic methods, such HIV-1 minority genomes can drive the evolution of the viral population and its further response to antiviral treatment[52].

Memory in viral quasispecies: history-dependent, persisting minority genomes

The presence of memory genomes as a result of quasispecies dynamics provided an interpretation for the origin of a class of minority genomes. Memory genomes are not the minority genomes that might be the result of coinfections followed by intrahost competition, nor those that result from reactivation as a consequence of a secondary infection or reinfection, but those that result from a prior selection event that influences the future composition of a single viral population. Because of its origins, memory is an asset of biological, complex replicative systems. The best characterized, classical memory system is the immune

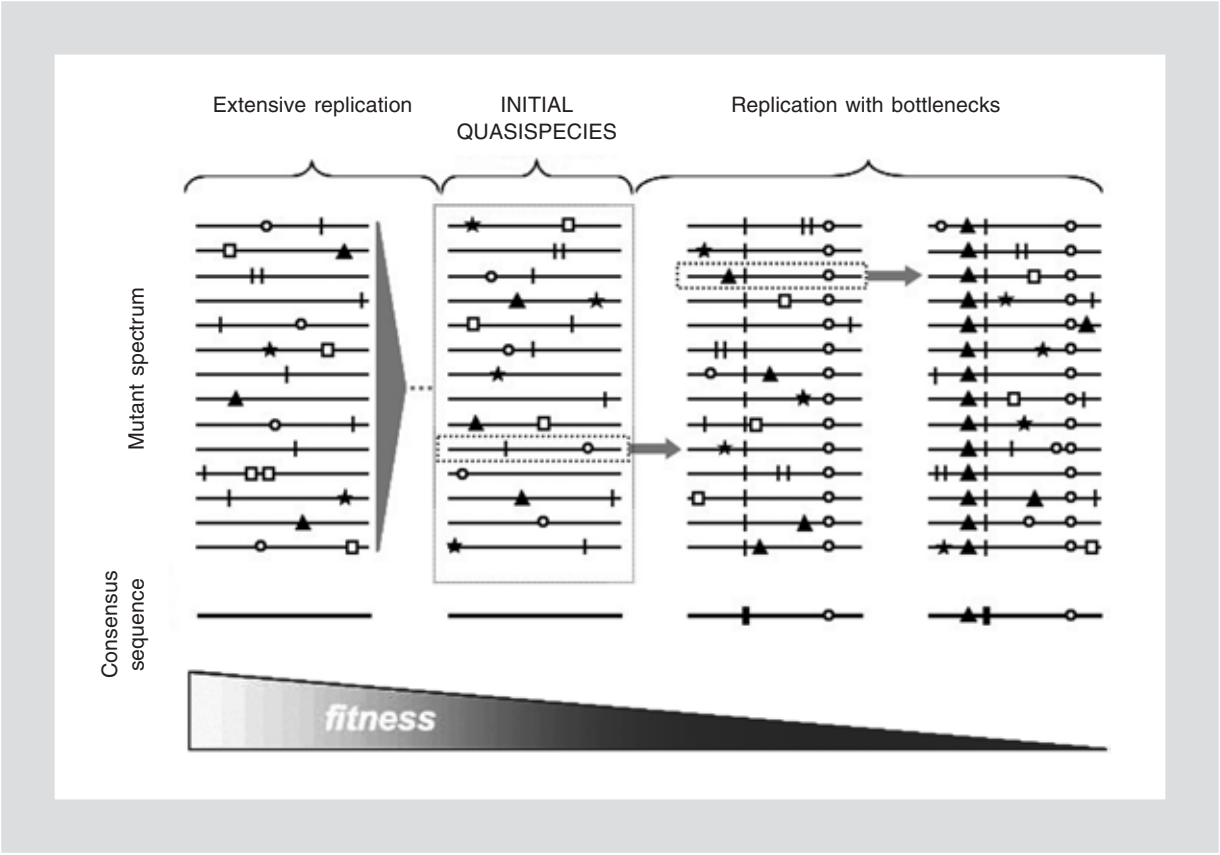


Figure 1. *Schematic representation of viral quasispecies dynamics. Individual genomes within the mutant spectrum are depicted as horizontal lines, and different mutations as symbols on the lines. The consensus or average sequence reflects the most abundant nucleotide at each position. The effect of replication regime on fitness variation is shown: large triangle represents the extensive replication of the quasispecies (equivalent to a large population passage in vitro) that originates a new quasispecies and generally results in fitness increase; small arrows indicate replication steps with consecutive bottlenecks (equivalent to plaque-to-plaque transfers in vitro) that result in the fixation of the mutations present in the parental genome and are usually accompanied by a fitness decrease. Bottleneck replication is produced* in vivo *when a selective pressure (e.g. in the form of antiviral treatment) is applied on the quasispecies (e.g. HIV-1), making the drug resistance mutations detectable in the consensus sequence of the virus.*

system of vertebrates, which includes subsets of T-cells, termed memory cells, capable of being amplified in response to an antigenic stimulus that had been experienced at an early phase by the organism (for a review of immunologic memory see[53]).

However, the term "memory" can have another meaning for viruses. Indeed, memory can also reflect the biological history of the virus that has shaped a genome organization, and its series of regulatory regions and encoded proteins. It is very difficult, if not impossible, to decipher the ancestral historical events that shaped viral genomes as we know them. Among other reasons this is because virology, as an experimental science, is only slightly more than one century old, and our capacity to define and compare viral (and cellular) genomes at the molecular level has been achieved only a few decades ago. This historical, long-term memory, as important as it is to understand virus origins[6], is not the objective of this review. It is the short-term memory in viruses, which is a consequence of quasispecies dynamics, that may have implications for viral diagnosis and treatment of the infected patient and that is summarized here.

How was quasispecies memory found? To test whether RNA virus quasispecies were endowed with short-term memory mechanisms, well-defined traits that could serve as memory markers, together with controlled evolutionary lineages, were needed. These requirements were fulfilled by an important ribovirus pathogen that has served as a model system for evolutionary studies: FMDV, a picornavirus with a naked capsid of icosahedral symmetry. A major antigenic site located at a mobile surface loop of one of its capsid proteins was the first marker chosen. A collection of neutralizing

MAb were produced and characterized with regard to the structure of the epitopes they define and the capacity of the virus to generate MAb-escape mutants. One antigenic variant selected with a specific MAb directed to this antigenic site was used for memory studies because it showed decreased fitness, and, as a consequence, it reverted to the wild-type sequence upon passage in cell culture in the absence of the antibody. If memory was present, the revertant population should contain and maintain within its mutant spectrum a higher level of the original antigenic variant than control populations. This was indeed found to be the case[48], and figure 2 displays schematically the main molecular events underlying FMDV memory, according to current experimental evidence. Memory in FMDV was also found with an independent marker, an internal oligoadenylate tract present in populations subjected to repeated bottleneck events. The internal oligoadenylate tract reverted upon passage of the virus in cell culture, and again it was found in a subset of memory genomes in the revertant population. This type of memory is termed "replicative memory", and its main features are summarized in table 1 (based on[6,48-50,52,54,55]).

From these experimental results, the relevant question raised was: What is the basis of quasispecies memory? In our view, the key event that determines this type of memory is the fitness increase experienced by the mutant genomes, that will later become memory genomes, when they replicate under the conditions (a specific selective pressure exerted for instance by a MAb or antiviral drug) that render such mutant genomes dominant (Fig. 2). Indeed, it has been documented that RNA genome replication in a given environment entails fitness gain, measured in that same environment[56,57]. Therefore, since the frequency or relative amount of subpopulations of viral genomes in the quasispecies depends on the relative fitness of such subpopulations, memory genomes (maintained after discontinuation of the selective pressure) will be present at a higher frequency than they had in the original population. In other terms, as belonging to the memory class, genomes will be found at frequency higher than they would have had in the absence of the selection-replication (fitness-increasing) step. This model of memory acquisition is consistent with the observation that memory levels are fitness-dependent, and that population bottlenecks erase memory[48,49], one of the distinctive features of quasispecies memory (see Table 1). A similar level of memory genomes can be maintained during many rounds of viral replication, and its gradual decrease followed a strikingly similar kinetics in several parallel lineages[54]. Therefore, memory defines a class of persisting minority genomes, unless population bottlenecks intervene. The memory levels in the FMDV lineages studied, defined either with an antigenic change or an internal polyadenylate as the memory marker, were 10- to 100-fold larger than the levels that could be attributed solely to mutational pressure (that are in the range of 0.01% considering an average mutation rate of 10^{-4} substitutions per nucleotide copied)[1,2].

When a viral population, with its memory genomes, replicates in the same environment in which memory was generated, both the dominant genomes and the memory genomes gain fitness in a rather parallel fashion[55]. This observation is in agreement with the Red Queen hypothesis applied to virus replication kinetics, previously documented with competing vesicular stomatitis virus populations[58]. As a consequence, memory genomes do not fade away immediately[49,54], but rather they can remain as a subpopulation of genomes in the evolving quasispecies, provided no population bottlenecks intervene. Thus, memory genomes may confer a selective advantage to a viral population by allowing the virus to respond more rapidly to a selective constraint that was previously experienced by the same evolutionary lineage. This is of consequence for the effectiveness of antiviral treatment, as discussed below for HIV-1 infections. In HIV-1, however, memory acquires new components, derived from the molecular events that characterize the lifecycle of retroviruses.

Molecular memory in HIV-1 *in vivo*

The replication cycle of retroviruses is characterized by an integrative phase as proviral DNA in the nucleus of the infected target cells. In the case of HIV-1, integrated copies of the viral genome constitute a reservoir of genetic variants that upon cellular activation, can contribute new mutant distributions to the circulating quasispecies. As a consequence, several types of long-lived infected cells (resting $CD4^+$ lymphocytes, macrophages and others) in different anatomical compartments (including lymph nodes and the central nervous system) are potential reservoirs for HIV-1[59-64]. Viruses in reservoirs can evolve more slowly than those circulating in the plasma of the same patient, since proviral DNA copies of HIV-1 genome replicate as cellular genes with the high fidelity copying inherent to cellular DNA polymerases endowed with proofreading activities and subjected to post-replicative repair

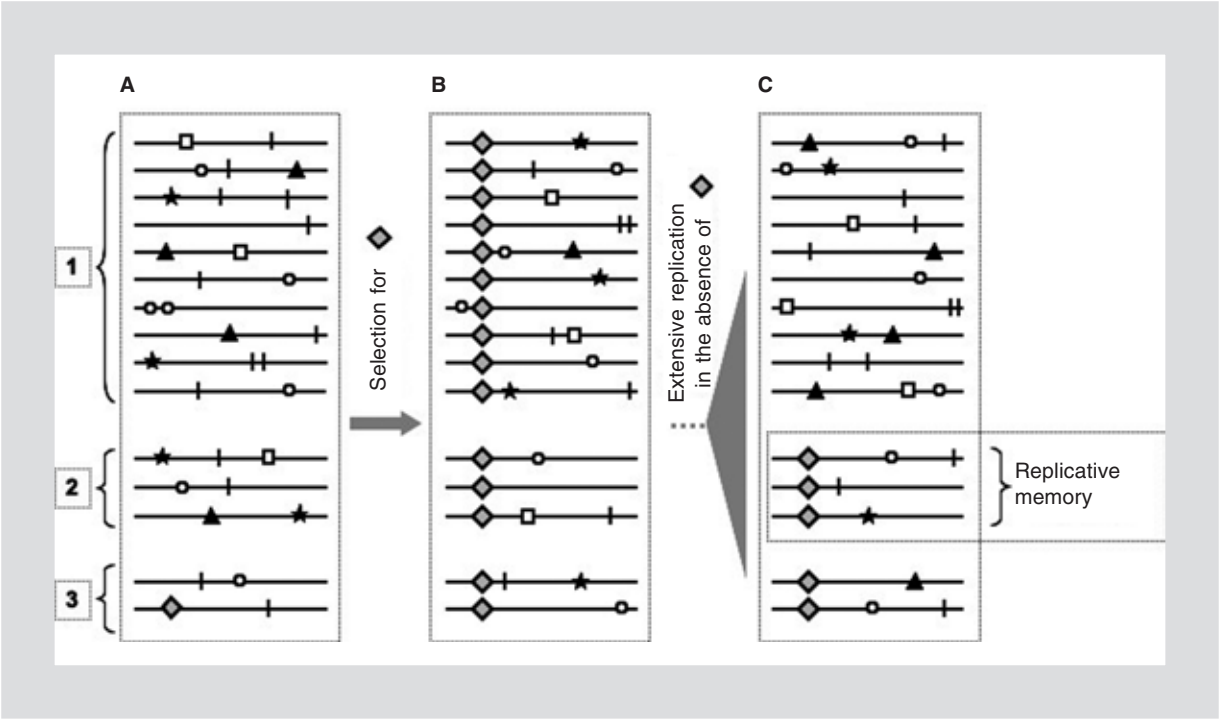


Figure 2. *Memory in viral quasispecies. The mutant spectrum of the quasispecies is divided into three frequency levels: 1, the most abundant or majority genomes; 2, minority components at frequencies ranging from about 0.1 to 20% of the total population; 3, quasispecies background with basal minority components at frequencies < 0.1%, derived from the mutational pressure (see text for details). Initially* ***(A)*** *a genetic marker (represented by a grey diamond) is present as a mutation encoded in a genome belonging to the basal components of the quasispecies. When this marker is selected (e.g. by an antiviral drug to which the mutation confers resistance), it becomes dominant* ***(B)*** *and the genomes encoding such mutation increase their average fitness, provided that the selective pressure is operating. If the drug pressure is discontinued and the quasispecies undergoes extensive replication, the mutants show a selective disadvantage with respect to the revertant wild-type genomes. However, since mutants encoding the genetic marker experienced a fitness increase while they were dominant, they may remain at level 2 as replicative memory genomes* ***(C)****. The detection of minority memory genomes in* ***(C)****, by means of an experimental method more sensitive than traditional genotyping techniques, informs about the variants that were dominant earlier in the same evolutionary lineage and are capable of driving its ensuing evolution.*

mechanisms[65,66]. In particular, integrated proviruses can maintain for months or years the genotypes, either wild-type or drug-resistant, exhibited when the reservoir was occupied. Therefore, HIV-1 genomes present in a reservoir can act as an archive of viral variants that may substantially differ from the genotype currently dominant in the plasma. This eventual discordance is magnified by two facts: reverse transcriptase (RT) inhibitors and other pre-integration drugs do not affect the integrated proviruses, and some anatomical sites may provide a barrier to the accessibility of certain antiretroviral drugs[62-64,67-70]. As a consequence, viruses from reservoirs can reemerge and perturb or occupy the circulating quasispecies when the selective pressures compromise the current plasma HIV-1 population[19,22,40,45,66,67,71]. The reemergence of viral sequences from cellular or anatomical reservoirs can be considered as a particular type of molecular memory of the past intrahost evolutionary history of the retrovirus, and we have called it "reservoir memory". Therefore, in HIV-1, two distinct types of molecular memory might operate *in vivo*: this reservoir memory, previously documented in several reports devoted to reemergence of viral variants, as well as a "replicative memory" analogous to that discovered in FMDV (described in the previous section) and eventually present in all RNA viruses as a natural consequence of their quasispecies dynamics.

In order to study the putative existence of these two memory mechanisms during HIV-1 infection *in vivo*, different cohorts of naive and pretreated patients were retrospectively analyzed. Among them, a detailed analysis was performed on selected subsets of patients undergoing HAART at the Hospital Carlos III (Madrid, Spain), whose well characterized treatment histories showed pronounced alterations in their viral load and/or $CD4^+$ count that could be related with quasispecies memory events. In all those cases, HIV-1 sequential samples

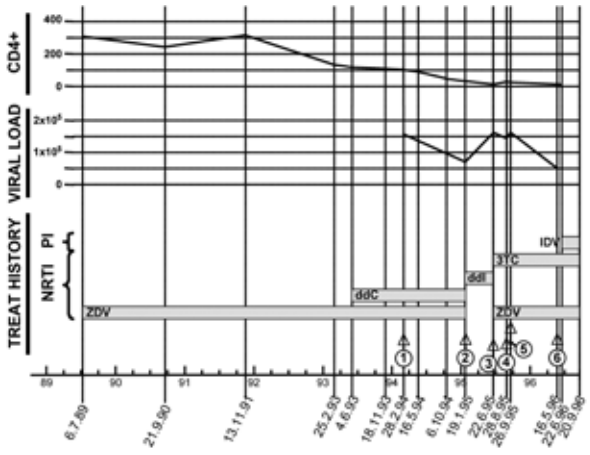
were collected, plasma viral RNA and/or proviral DNA was extracted, selected regions from the *pol* and *env* genes were amplified by high fidelity RT polymerase chain reaction (RT-PCR) or PCR, and sequenced as previously described[28,29,50,72]. Among them, four infected patients with unusual responses to their treatment histories were chosen for further HIV-1 genetic and phylogenetic analyses.

The first two cases analyzed were examples of reservoir memory in the intrahost HIV-1 evolution[50], with features analogous to those previously reported for viral reemergence[19,40,66,67,71]. In turn, the third and fourth cases documented for the first time the implication of replicative memory in HIV-1 evolution *in vivo*, associated with the maintenance of minority genomes within the mutant spectrum of the quasispecies[50-52].

Reservoir memory was investigated by means of a phylogenetic analysis of consensus sequences obtained at different time points of the treatment history (detailed in[50]). Case 1 documented the reemergence of wild-type HIV-1 genomes, lacking RT inhibitor resistance mutations such as the multi-nucleoside-resistance insertion T69SSS[28,73-76] that had been progressively accumulating in the circulating quasispecies. In this case, the mobilization of wild-type viruses from cellular or anatomical reservoirs was probably triggered by the introduction of a new drug combination, including a protease inhibitor (PI) to which the insertion-containing HIV-1 genome dominating in the plasma was susceptible and less fit than the ancestral genome. The analysis of a second patient (Case 2) revealed that reemergence of drug-resistant HIV-1 genomes from reservoirs can occur in two consecutive rounds, as a result of treatment interruptions or bad compliance to the prescribed drug combination. This mechanism of intrahost HIV-1 evolution based on the reemergence of genomes from reservoirs has been thoroughly analyzed[19,22,40,45,50,66,67,71] and will not be further discussed in the present review.

In turn, the discovery and clinical implications of the second kind of memory, replicative memory, in HIV-1 quasispecies[50,52] deserve a more detailed discussion here. To evaluate the eventual presence of replicative memory at different points of the selected treatment histories, besides the analysis of consensus sequences a detailed phylogenetic analysis was performed on sequential clonal sequences (from 20 to 30 from each sample analyzed). Following this systematic approach, the first patient where replicative memory was found was Case 3, a hemophiliac man born in 1970, infected at the age of 12 after receiving contaminated blood products and subject to antiviral therapy since 1989 until his death in 1996[50]. Figure 3a shows a schematic representation of his treatment history, together with his plasma viral load and $CD4^+$ lymphocyte count evolution and the time points (1 to 6) where consensus and clonal sequences were obtained. The analysis of RT consensus sequences (Fig. 3b) showed that certain HIV-1 key mutations related to decreased sensitivity to RT inhibitors were maintained at all time points. Nevertheless, dominance of genomes encoding the multidrug resistance (MDR) insertion T69SSS[28,73-76] was lost within a six month interval (from 1.95 to 6.95), probably as a result of the switch from azidothymidine plus zalcitabine (AZT/ddC) treatment to didanosine monotherapy. The reintroduction of AZT (in combination with lamivudine) was associated with the selection, two months later (sample 4), of a virus whose consensus sequence contained the RT insert with the genotype T69SSG. A phylogenetic analysis was performed with 120 clonal sequences obtained from samples 1 to 6 (Fig. 3c), showing a clear, statistically supported separation among sequences harboring the dipeptide insertion (either SS or SG) and those without any RT insertion. Interestingly, sample 2, which together with sample 1 had a RT consensus sequence with T69SSS genotype (Fig. 3b), contained one clone (number 2-17, representing 5% of the total quasispecies) without RT insertion. The minority genomes represented by this clone originated the majority of clones (85%) in the next sample (sample 3). In turn, sample 3 showed a consensus sequence without RT insertion, but harbored three clones (numbers 3-3, 3-5, and 3-8, representing 15% of the total population) with a T69SSS genotype. Such minority sequences from sample 3 were likely selected by the reintroduction of AZT (together with lamivudine) and restored a quasispecies dominated by viruses with the MDR insertion in samples 4, 5, and 6. Therefore, during the treatment history of Case 3, minority genomes without insertion were maintained as replicative memory in populations dominated by insertion-coding genomes and, conversely, minority memory insert-containing genomes were present in populations dominated by viruses devoid of the insertion (Fig. 3c). It must be underlined that in samples 2 and 3 the analysis of their consensus sequence, the conventional method for guiding antiviral treatment in the clinical setting, did not show any evidence of the existence of minority memory genomes hidden within the mutant spectrum. Such minority components maintained in the population as a record of the previous stages, and responsible for the ensuing intrahost

A



B

Sample (date)	RT amino acid position (codons 40-220)																	
	41	46	53	61	68	**69**	**ins.**	83	88	127	135	166	177	186	196	202	214	**215**
	M	K	E	F	S	**T**	**-**	R	W	Y	T	K	D	D	G	I	L	**T**
1 (28.2.94)	**L**	-	-	-	-	**S**	**SS**	K	-	F	I	R	N	-	E	-	F	**Y**
2 (19.1.95)	**L**	(E)	(G)	(S)	-	**S**	**SS**	K	S	F	I	R	-	Y	E	V	F	**Y**
3 (22.6.95)	**L**	-	-	(S)	-	**S**	-	K	-	F	I	R	-	-	E	-	F	**Y**
4 (28.8.95)	**L**	-	-	-	(N)	**S**	**SG**	-	S	-	I	R	E	-	E	-	F	**Y**
5 (26.9.95)	**L**	-	-	-	-	**S**	**SG**	-	S	F	I	R	E	-	E	-	F	**Y**
6 (16.5.96)	**L**	-	-	-	-	**S**	**SG**	-	S	F	I	R	E	-	E	-	F	**Y**

Figure 3. *Clinical data, consensus sequences, and phylogenetic analysis of clonal sequences of Case 3.* ***A:*** *treatment history, evolution of CD4+ count (CD4+ lymphocytes/ml) and viral load (HIV-1 RNA copies/ml). Dashed boxes delimit the periods of treatment with the different inhibitors. Sampling dates are written as day.month.year. Sample identification numbers of the six samples subjected to sequence analysis (see details in the text) have been encircled.* ***B:*** *amino acid substitutions found in consensus sequences of the RT (codons 40-220) of sequential HIV-1 samples. The single letter amino acid code is used. Dash means no change relative to the reference HIV-1 sequence shown at the top (HIV-1 HXB2 strain). Amino acid substitutions at positions relevant to resistance to PI or RT inhibitors have been highlighted with bold-face letters. Different amino acids at one position indicate that the residue is found in a mixture in the PCR fragment obtained as population sequence. NRTI, nucleoside reverse transcriptase inhibitors; 3TC: lamivudine; ddC: zalcitabine; ddI: didanosine; ZDV: zidovudine; PI: protease inhibitors; IDV: indinavir; RT: reverse transciptase.*

evolution, can only be revealed through the clonal analysis retrospectively performed[50] or, eventually, by any other technology capable of detecting minority genomes in complex samples (see next section).

Following an analogous approach, an additional case of HIV-1 replicative memory was detected and analyzed[52]. Case 4 was a man born in 1956, infected through homosexual intercourse some time before 1997. His well documented treatment history, starting in 1997, showed large fluctuations in viral load and CD4+ count associated with two periods of treatment interruption. It had been previously described that re-emergence events, such as reseeding of HIV-1 reservoirs and activation of proviral DNA during treatment interruption, could compromise the response upon reintroduction of treatment[40-42,44,45,77-79]. Figure 4a shows the clinical parameters and treatment history of Case 4, as well as the time points (1 to 8) where consensus and clonal sequences were obtained. Consensus sequences corresponding to the RT (Fig. 4b) showed that a MDR genotype associated with the Q151M complex[80-83] was present in samples 1 to 6, with the exception of sample 2 that showed a RT inhibitor-susceptible genotype. Subsequently, 209 clonal sequences (involving 29-32 clones from each sample 1-7), all of them including a 1,083 bp-long fragment of the *pol* region, were aligned and analyzed. In sample 2, despite showing a consensus sequence without any substitution

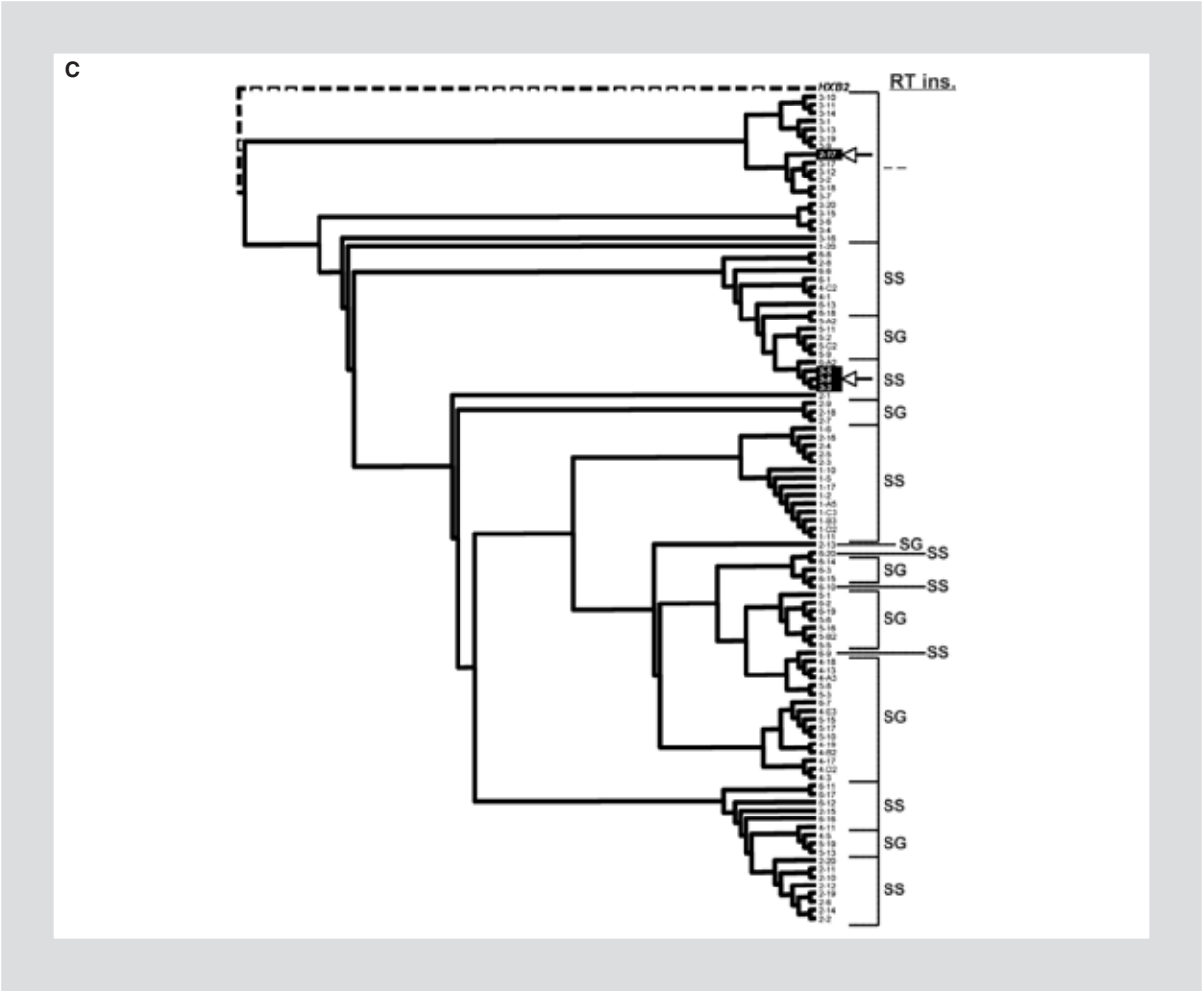
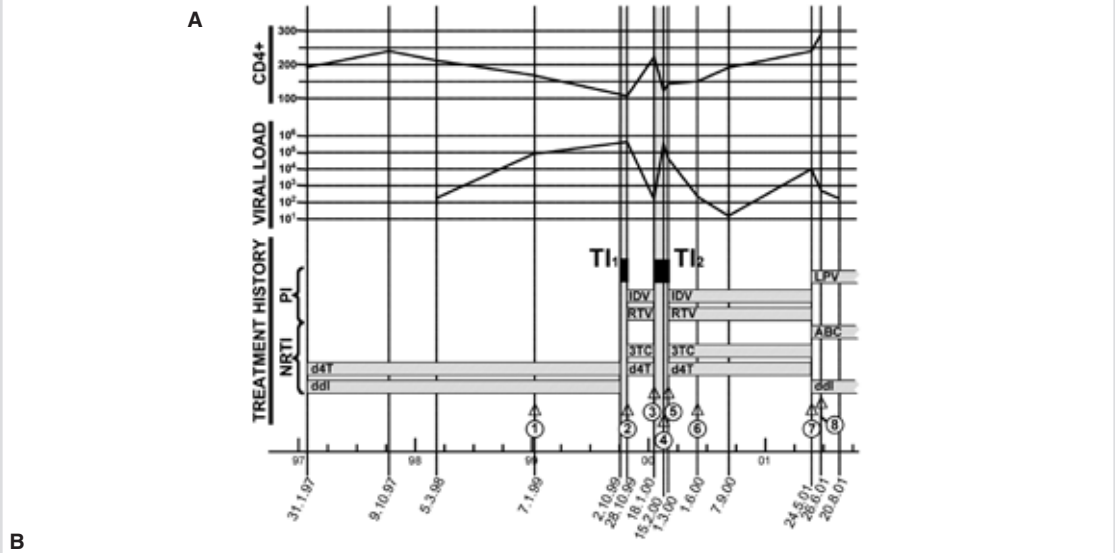


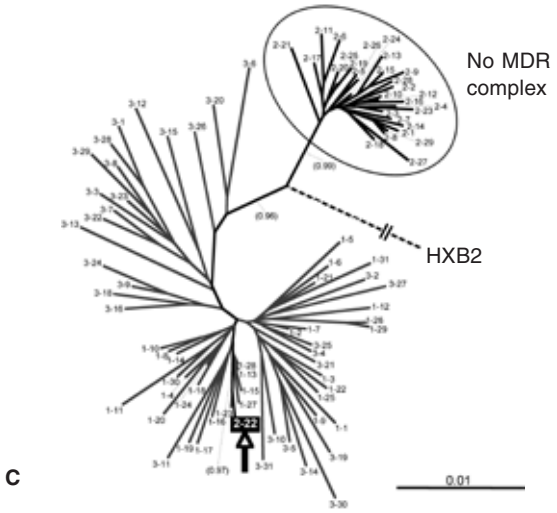
Figure 3. ***C:*** *Maximum Parsimony clustering of the 120 clonal sequences obtained from samples 1-6, using HIV-1 HXB2 as reference sequence. Unique sequences are identified as "sample-clone number". Groups of clones with identical sequences are labeled as "sample-letter-number", as detailed in[49]. The nature of the two-amino acid insertion between codons 69 and 70 of the reverse transcriptase is shown (SS: genotype T69SSS; SG: genotype T69SSG; --: genotype T69S). Minority memory genomes present in samples 2 (represented by the sequence 2-17, without insertion) and 3 (3-3, 3-5 and 3-8, with a SS insertion) are highlighted and marked with arrows.*

related to drug resistance, one out of 29 clones (clone 2-22, representing 3.5% of the mutant spectrum) harbored the MDR complex 75I/77L/116Y/151M[52]. The genomes represented by this sequence had been maintained during the four-week treatment interruption (from 2.10.99 to 28.10.99) as replicative memory in circulating quasispecies, after being dominant (100% of the mutant spectrum) in sample 1. Three months later, genomes derived from those represented by clone 2-22 constituted the dominant genomes (83.3%) in sample 3. Then, a second six-week treatment interruption produced additional fluctuations in the quasispecies, leading to different proportions of genomes harboring Q151M-associated substitutions. A phylogenetic analysis of the clonal sequences (Fig. 4c) showed that minority clone 2-22 clustered within a clade formed by clones from samples 1, 3, 4, and 6. The remaining 28 clones from sample 2 contributed a compact and statistically supported group, far from the position occupied by sequence 2-22. Apart from other considerations regarding the clinical evolution of this patient (previously discussed[52]) it must be underlined that this report documented for the first time that in a treatment interruption context, replicative memory maintained in the form of minority genomes within the quasispecies can play a major role in the evolution of HIV-1 quasispecies after resumption of therapy. Indeed, Case 4 confirmed the clinical implications of replicative memory, as described for Case 3, and showed that viral genomes encoding any of the



B

Sample (date)	RT amino acid position (codons 40-220)																				
	49	50	**67**	**68**	**75**	**77**	83	**116**	**151**	163	165	169	173	**184**	188	195	196	200	204	207	**211**	214
	K	I	**D**	**S**	**V**	**F**	R	**F**	**Q**	S	T	E	K	**M**	Y	I	G	T	E	Q	**R**	L
1 (7.1.99)	R	V	-	**G**	**I**	**L**	K	**Y**	**M**	-	I	D	-	-	-	L,I	-	A	-	E	**K**	F
2 (28.10.99)	R	V	-	-	-	-	K	-	-	-	I	D	K,A	-	-	-	-	A	-	E	**K**	F,L
3 (18.1.00)	R	V	-	**G,S**	**I**	**L,F**	K	**Y**	**M**	-	I	D	-	-	-	L	-	A	-	E	**K**	F
4 (15.2.00)	R	V	-	**G**	**I**	**L,F**	K	**Y**	**M**	T,S	I	D	-	-	-	L,I	-	A	-	E	**K**	F
5 (1.3.00)	R	V	-	-	**V,I**	-	K	**Y**	**Q,M**	S,T	I	D	-	-	-	L	-	A	-	E	**K**	F
6 (1.6.00)	R	V	-	**G,S**	**I,V**	**L,F**	K	**Y,F**	**Q,M**	S,T	I	D	-	-	-	L,I	G,E	A	-	E	**K**	L,F
7 (24.5.01)	R	V	**D,A**	-	-	-	K	-	-	-	I	D	-	**V**	-	L	-	A	-	E	**K**	-
8 (26.6.01)	R	V	-	**G**	**I**	**L**	K	-	-	T	I	D	-	-	C	L	-	A	K	E	**K**	-



C

Figure 4. *Clinical data, consensus sequences, and phylogenetic analysis of clonal sequences of Case 4.* ***A:*** *treatment history, evolution of CD4⁺ count (CD4⁺ lymphocytes/ml) and viral load (HIV-1 RNA copies/ml). Dashed boxes delimit the periods of treatment with each drug. Two treatment interruptions (periods TI_1 and TI_2) are shadowed. Sampling dates are written as day.month.year. Sample identification numbers of the eight samples subjected to sequence analysis (see details in the text) have been encircled.* ***B:*** *amino acid substitutions found in consensus sequences of the RT (codons 40-220) of sequential HIV-1 samples (see details in the legend of Fig. 3b).* ***C:*** *Neighbour-Joining tree of the 90 clonal sequences corresponding to samples 1-3 (adapted from[51]). Clone numbers are shown, and minority memory subpopulation present in sample 2 (represented by the sequence 2-22, harboring the multidrug resistance (MDR) complex Q151M) is highlighted and marked with an arrow. Bootstrap values relevant for clonal sequences from sample 2 are indicated in parenthesis. The bar represents 0.01 substitutions per nucleotide. ABC: abacavir; d4T: stavudine; LPV: lopinavir; RTV: ritonavir; IDV: indinavir; 3TC: lamivudine; ddI: didanosine.*

two main multi-nucleoside-resistance clusters of mutations can be maintained in the form of minority memory genomes not detectable by consensus sequencing of the quasispecies.

In parallel to the phylogenetic analyses performed, a mathematical model was developed to test if reservoir and/or replicative memory could emerge as an evolutionary strategy of HIV-1 under antiviral pressure. The in silico model was based on the theory of quasispecies[3-5] and considered two viral components (typically, wild-type and drug-resistant mutant) competing in the circulating quasispecies, together with a third component capable of reemerging from a latent reservoir after activation by a certain biological stimulus. The model system was numerically and analytically solved for switches in the drug combination[50] and treatment interruptions[52], and showed a good agreement with the experimental data regarding the maintenance of minority genomes within intrahost HIV-1 evolution. Therefore, both experimental and theoretical approaches show that quasispecies memory can be considered as a general strategy in HIV-1 dynamics, and encourage the systematic search for minority memory genomes in candidate cases. Figure 5 depicts an interpretation of how HIV-1 moves through the accessible sequence space of the quasispecies as a consequence of antiviral drug pressure, and how, during such intrahost evolution, minority subpopulations can be maintained as a replicative memory that remains hidden to the traditional genotypic techniques usually available in the clinical setting.

Several complicating factors render the prediction of memory levels for HIV-1, and for any virus, very difficult. One such factor is the dependence of fitness values on environmental conditions. Drug resistance may entail a fitness cost, which may be compensated by subsequent replication rounds when the population size is large[84]. Furthermore, fitness of a drug-escape mutant may depend on the presence of other antiviral drugs, as it has been well documented for HIV-1 (reviewed[85]). The influence that a specific mutation can have on fitness might depend on the sequence context of the viral genome, on the collective behavior of the quasispecies as a whole, and it may be altered by the unceasing effects of HIV-1 mutation and recombination. Therefore, it is a formidable task to predict memory levels *in vivo*, despite their documented and expected dependence on the initial fitness of the genomes destined to become memory[49].

The distinction between replicative and reservoir (or anatomical) memory that might seem straightforward for retroviruses, may in some cases be fuzzy. The selection-competition or selection-reversion cycles that lead to memory may occur with widely different kinetics in separate host compartments. In some of them, replication may be slow enough so as to retain certain genomes in a quasi-frozen state, relative to other compartments (that is the reason why reservoir memory has

Table 1. Main features of viral quasispecies and replicative memory

Viral quasispecies
A complex mutant spectrum may favor adaptability of the ensemble. (Relevant parameters are: mutation frequencies within mutant spectra, virus population size, genome length, and number of mutations required for a phenotypic change).
Positive interactions (complementation) and negative interactions (interference) can occur among components of the mutant spectrum.
The collective behavior of a viral quasispecies, through complementation or interference, can affect disease progression, response to antiviral treatments, and survival versus extinction of the viral population.
Quasispecies replicative memory
Memory genomes are descendant from the genomes that were dominant earlier, in the same evolutionary lineage.
The presence of minority memory genomes is expected from viral quasispecies dynamics. Upon selection, a mutant virus replicates and, therefore, increases its fitness. When it reverts, its level in the population is higher than expected from mere mutational pressure exerted on the parental genomes.
Memory levels are dependent on the relative fitness of the virus destined to become memory. (In the model studies with foot-and-mouth disease virus a 7.6-fold higher fitness resulted in 30- to 100-fold higher memory level).
Different memory levels can be attained for different genetic markers, depending on mutation rates and relative fitness values. In the quasispecies analyzed, biologically relevant memory levels are present at frequencies between 0.1 and 20% of the total number of genomes in the population.
Upon viral replication, memory genomes gain fitness in parallel with the majority genomes of the same population (Red Queen hypothesis).
Population bottlenecks erase memory.
Memory levels may decrease as virus replicates.
Memory genomes can affect the evolutionary outcome of the quasispecies.

Based on[6,47-49,51,53,54].

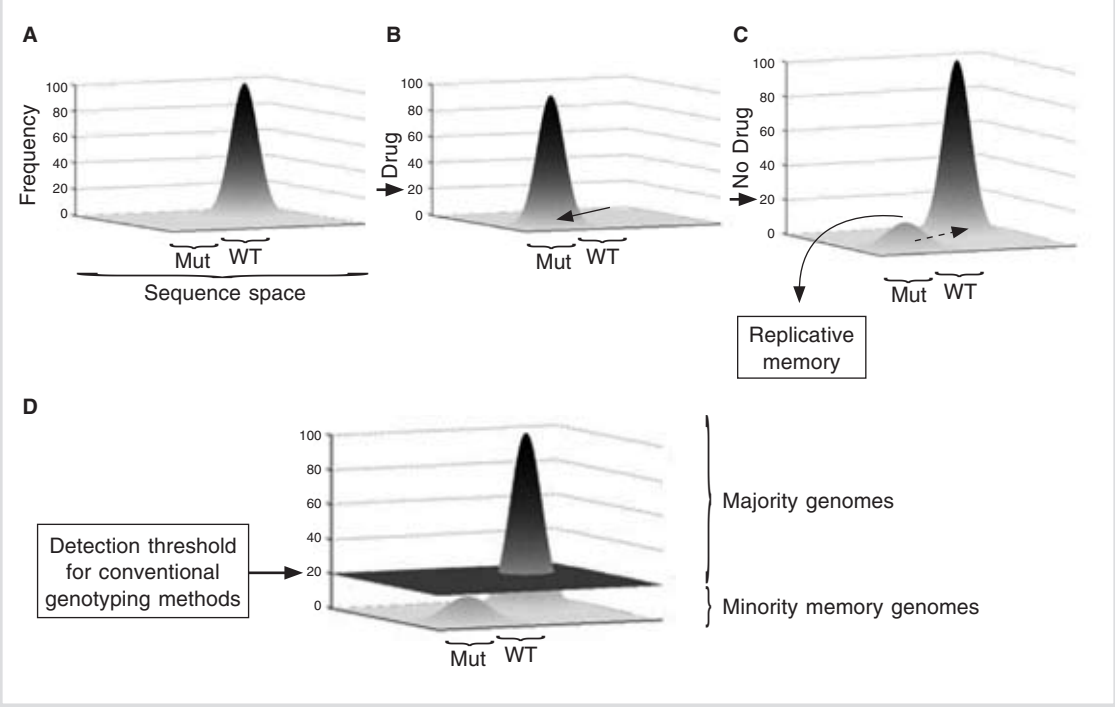


Figure 5. *Graphic interpretation of the HIV-1 dynamics and the generation of replicative memory as a result of the antiviral selective pressure. An HIV-1 wild-type population* ***(A)*** *moves through the sequence space of the quasispecies to respond to the treatment pressure (arrow labeled 'Drug') until it reaches a region characterized by a drug-resistant mutation* ***(B)****. During stage* ***(B)****, drug-resistant mutants acquire compensatory mutations and increase their average fitness (see text and Table 1 for details). If the drug pressure is discontinued (arrow labeled 'No Drug'), the competition between the drug-resistant population and the high-fitness wild-type mutants generated favors the recovery of a wild-type genotype, although minority memory genomes can be maintained in the quasispecies as a record of the past evolutionary history* ***(C)****. In this situation, if the same, or similar, drug pressure is reintroduced, a quick restoration of the drug-resistant population (B) will be produced. Panels* ***(A)*** *to* ***(C)*** *are equivalent to those depicted in figure 2. Since the detection threshold for conventional HIV-1 genotyping methods, such as consensus sequencing and line probe assay hybridization, is about 20% of the total population, represented by a horizontal plane in* ***(D)****, they can only identify the majority or most frequent genomes, while minority genomes remain hidden. Therefore, if the quasispecies is analyzed by genotypic methods currently used in the clinical setting, the situation represented in* ***(C)*** *will be considered identical to that depicted in* ***(A)****, and a pure wild-type genotype would erroneously be assumed. Mut: mutant; WT: wild-type.*

sometimes been called "non-replicative" memory[50,86]), yet they may be subjected to the same replicative events that lead to replicative memory. The relationship between replicative and reservoir memory was addressed in a theoretical model that supported the presence of the two classes of memory[50].

The results reviewed in this section show that minority memory genomes present at levels as low as 3% can influence the ensuing intrahost evolution of HIV-1 *in vivo*. Further research is required to determine at what point a minor drug-resistant genome may become clinically relevant. Since the replication of virus quasispecies is characterized by mutation rates with an average of 10^{-4} substitutions per nucleotide copied[1,2], it has been predicted that all HIV-1 individual mutations naturally occur at a frequency of about 0.01%[87], constituting the so-called "quasispecies background", as discussed for FMDV in the previous section. Therefore, only those memory genomes that are maintained at higher frequencies are likely to be biologically meaningful. With all the limitations summarized in the previous paragraphs, our analysis of different viral quasispecies suggests an upper level for memory genomes in the range of 20% of the total quasispecies (Table 1; Fig. 2), and a lower level between 1 and 0.1%. These upper and lower thresholds could define a putative interval, which includes most of the reported cases of persisting minority variants in HIV-1[19,21-47], where the minority genomes should be taken into account for designing individualized therapies. Nevertheless, systematic studies are required in

order to reach a consensus about the clinically relevant cutoffs for the detection of minority genomes in HIV-1 quasispecies. They will rely on the optimization and standardization of some of the ultrasensitive experimental methods that are currently available for the detection of low-abundance genomes.

Experimental methods for the detection of minority genomes within HIV-1 quasispecies

Evolution of traditional genotyping techniques

Although disregarded some years ago, it is currently accepted that the detection and characterization of minority genomes (among them, the minority memory genomes derived from the previous intrahost evolution) present in HIV-1 at a level higher than the quasispecies background is required in order to make viral genotyping a predictive tool of subsequent evolution under antiretroviral therapy. In fact, the successful characterization of minority genomes could open the door to a personalized clinical management of the infected patient. In this framework, the usefulness of traditional genotyping techniques is largely compromised. Population or consensus HIV-1 sequencing provides information limited to the genotype of the predominant or major viral variant, and it does not recognize minority subpopulations represented in less than 25 or 30% of the total quasispecies[19,88-91]. This detection sensitivity is only slightly improved by other genotyping techniques available in the clinical setting, such as those based on the hybridization of the labeled, RT-PCR-amplified viral genome to a limited number of probe oligonucleotides immobilized onto a nitrocellulose strip. Indeed, several reports have documented that these line probe assays (LiPA) cannot detect minority genomes representing less than 20% of the amplified population[92-95].

Traditionally, the investigation of the mutant spectrum of HIV-1 quasispecies in molecular biology laboratories has involved the sequence analysis of a representative number (in general, 10-30) of molecular clones derived from the amplified viral population[24-27,29,30,42,45,50,52,66,96].Nevertheless, this is a very expensive and time-consuming method that would require the analysis of 1,000 molecular clones to characterize the quasispecies at a resolution of 0.1%. In order to partially overcome this limitation, an alternative approach has been recently described that allows the straightforward characterization of minority genomes within HIV-1 quasispecies with an unprecedented level of detection (in the range of 0.005% of the amplified total population), involving the analysis of less than 100 molecular clones[97]. It is based on the progressive amplification of minority variants present in the viral quasispecies using specifically designed sets of specific PCR primers, followed by the clonal analysis of the selected subpopulation. This method has been termed "quasispecies diving" to metaphorically indicate that the successive PCR amplification protocol allows for the progressive approach to reach smaller, minority or "deeper" genomes in the mutant spectrum of the quasispecies. This notwithstanding, it is assumed that all the experimental methods for the detection of minority genomes based on clonal analysis of the population are unrealistic approaches for most clinical laboratories.

As an alternative, novel methodologies based on rapid and highly sensitive assays have been developed during the last decade and are currently available, though in non-commercial format, to the community of HIV-1 clinical research. Oligonucleotide ligation assay (OLA), based on selective hybridization followed by a confirmatory ligase reaction, was one of the earliest of such methodologies and shows a sensitivity of 5% for minority mutations[95,98,99]. Different variants of the heteroduplex mobility (HMA) and heteroduplex tracking (HTA) assays have been used to detect low-abundance subpopulations comprising 1-5% of the viral quasispecies[100-103]. Moreover, the quick development of allele-specific real-time quantitative PCR (ASPCR) technology has allowed the characterization of minor subpopulations (in the range of 0.05 to 1%) of drug-resistant HIV variants both in seroconverters and in pretreated patients[33,34,37,39,41,43,44,95,104-106].

Additionally, sequence-based methods have quickly evolved during the last decade, and they can currently be adapted to the detection of minority genomes in complex mixtures such as viral quasispecies without the need for a previous molecular cloning. In a recent example relevant to the HIV-1 clinical research, it has been shown that parallel allele-specific sequencing (PASS) allows the detection of minority HIV-1 drug-resistant variants present at levels ranging from 0.01 to 0.1% of the sample[36].

Ultra-deep pyrosequencing

A spectacular advance has been produced with the recently developed technique called "pyrosequencing"

that allows up to 400,000 individual reads per sequencing run, with an average length of 100-250 base pairs each[107]. This revolutionary technology is less laborious and less costly than clonal sequencing because all the process is automatic, and conventional procedures such as PCR amplification and bacterial transformation are not required. Being an unbiased sequencing-based strategy, it informs about all the mutations and single nucleotide polymorphisms (SNP) present in the region scanned, and not only about those positions previously selected, as is the case in the genotyping DNA microarrays described in the next section. Several relevant applications of ultra-deep pyrosequencing in virology have been documented, including the detection of lamivudine resistance mutations in HBV[108], the typing of human hantaviruses[109], the monitoring of resistance to adamantanes among circulating influenza A viruses[110], and the discovery of a new arenavirus related to lymphocytic choriomeningitis viruses[111].

Regarding HIV-1, this technology was soon adapted to the detection of PI resistance mutations[112] and, among other applications, it has allowed the massive analysis of viral integration site selection into host cell DNA[113]. Relevant for this review, ultra-deep pyrosequencing has been successfully used in control HIV-1 mixtures and clinical plasma samples for the simultaneous detection of minor drug-resistant variants in the HIV-1 *pol* gene at proportions as low as 5%[114] or even 2%[95,115] of the population. In order to generalize its use for HIV-1 genotyping, this technology still needs substantial improvements related to the short length of the sequence reads (currently limited to < 300 bp) and the high error rate associated to the pyrophosphate-based sequencing process (in the range of 5×10^{-3} to 10^{-2}, or 5-10 errors/Kbp)[115,116]. Nevertheless, computational methods have been recently described with which the frequencies of different HIV-1 variants are estimated, and intrahost population structures can be inferred[116]. Pyrosequencing technology is extremely powerful, and likely it will soon offer an unprecedented opportunity to characterize the subpopulation diversity within any viral quasispecies and other clinically relevant, complex genetic populations such as intrahost mitochondrial DNA or heterogeneous mixtures of cells from tumoral tissues[117,118].

DNA microarrays and nanotechnology-based biosensors

In parallel with the sequencing methodologies, an outstanding development has occurred during the last decade in the hybridization techniques of nucleic acids, together with innovations in chemistry and nanotechnology that allow the immobilization of different kind of molecules onto activated surfaces. This has led to the construction of a growing number of bio-affinity biosensors, among them the so called “microarrays” or “biochips”. The DNA microarrays are based in the possibility to covalently immobilize thousands of single-stranded DNA probe oligonucleotides on a solid (generally, chemically modified glass) substrate, in arrays of points of about 100 micrometers in diameter. Target molecules present in a natural sample are fluorescently labeled, and their specific hybridization with the surface-bound complementary probe is detected by means of high-resolution scanning[119-122]. This technology has been extensively applied to biomedicine, including the following research lines: i) studying the gene expression profiles in organisms (e.g. related with a pathogenic state or with an infection process); ii) genotyping and SNP mapping in cellular or viral genes; iii) re-sequencing of genomes; iv) detection of pathogenic microorganisms in natural samples (for a review, see[123]).

Relevant examples of the usefulness of DNA microarray technology in virology include the detection, typing or subtyping of influenzaviruses[124-126], hepatitis viruses and/or HIV[127-129], rotaviruses[130], or other viruses[131-134], the characterization of recombination in polioviruses[135], and the determination of secondary RNA structure in HIV-1 and HCV genomes[136-137]. Especially relevant for this review is the applicability of DNA microarrays for viral genotyping, in which the detection of fluorescence in one point of the array means that the labeled target genome harbors the sequence fully complementary to the probe oligonucleotide immobilized at that position. In turn, all the non-perfect double-stranded DNA hybrids, even those involving a single point mutation, are washed and do not produce a positive signal. This technology allows the characterization of a large number of mutations and SNP in a single hybridization experiment, as documented for HIV-1 and other viruses[126,131,134,138-140]. Genotyping DNA microarrays constitutes a very promising technology for the characterization of minority genomes in HIV-1 quasispecies, including the memory genomes derived from the previous intrahost evolution in the patient. Indeed, this technology allows the detection of minority genomes in a complex mixture even at frequencies as low as 1%[135,141] or even 0.1% if microarrays are combined with mass spectrometry[139,142,143].

The DNA microarrays have proved to be very versatile biotechnology tools, and are less costly than clonal analysis or ultra-deep pyrosequencing for high-throughput viral genotyping. Nevertheless, HIV-1 genotyping DNA microarrays useful in the clinical setting must be carefully designed in order to minimize the main limitation of this technology: due to the genetic variability of HIV-1 and other RNA viruses, clusters of adjacent mutations can be present in relevant regions of functional viral genes, interfering with the accuracy of probe-target hybridization. This requires the design and testing of redundant sets of probe oligonucleotides covering the (previously known or predicted) variability of the nucleotide positions flanking the interrogated one, as well as the use of bioinformatic tools to distinguish meaningful hybridizations from partially mismatching duplexes that could render false-positive or false-negative signals. Moreover, together with probe oligonucleotides harboring different variants of point mutations involved in resistance, DNA oligos containing genomic regions with insertions and deletions relevant for HIV-1 drug-resistance must be included in the array. Additionally, the worldwide clinical usefulness of HIV-1 genotyping microarrays will require the inclusion of such a complete set of DNA oligonucleotide probes complementary to the selected genomic regions present in B and all non-B subtypes, as well as in circulating recombinant strains.

From a technological point of view, despite its broad applicability in different areas of biomedicine, DNA microarrays face a limitation related to the need for fluorescent labeling of the sample to be analyzed. The DNA labeling usually requires an extra and expensive pretreatment of the sample, and its efficiency imposes a limit for the sensitivity and accuracy of the microarray technology. Therefore, during the last decade, several types of alternative, nanotechnology-inspired detection techniques have been developed in order to dispose of simple, fast, label-free biosensors. Essentially, the techniques that avoid fluorescent labeling of the target DNA are either sensitive to mass increases, such as those occurring upon hybridization, or to certain chemical signatures of specific molecular groups, which change or appear upon hybridization (reviewed[144]). For instance, one of the recent achievements in the field of nanotechnology-based biosensors has allowed the label-free, specific detection of DNA hybridization at room temperature[145]. It is based on the hydration-induced tension on nucleic acids bound to functionalized cantilevers, an ultrasensitive effect that allows the genotyping of target molecules even at femtomolar concentration, and the detection of low abundance genomes even at frequencies of 0.1% of the total population. This is only an example of the kind of technologies currently available that show potential applicability for the detection of minority genomes in viral quasispecies and could be adapted to the clinical setting in the near future.

Conclusion

Viral quasispecies such as HIV-1 are complex replicative systems, evolving as an ensemble of interactive variants, which together contribute to viral pathogenesis. The HIV-1 quasispecies can be endowed with a molecular memory of their past intrahost evolutionary history, maintained in the form of minority components within the mutant spectrum. They are capable of re-emerging rapidly and becoming a majority if the evolving viral population is subject to selective pressures, which are the same or similar as those present when the HIV-1 memory genomes were selected. This fact is particularly relevant for antiviral treatment since several reports have documented that HIV-1 may harbor minority or low-abundance drug-resistant variants that can quickly expand under drug selection pressure and compromise the treatment efficiency. Apart from the examples of HIV-1 reservoir and replicative memory reviewed here and the data reported by other groups regarding persisting minority HIV-1 variants, additional studies using retrospective plasma or peripheral blood mononuclear cell samples obtained from patients with well-characterized treatment histories and clinical outcomes are needed. They will establish the extent to which the maintenance of minority memory genomes within the mutant spectrum can be operating as a general evolutionary strategy used by HIV-1 under antiviral pressure. Also, systematic and statistically supported studies are required in order to reach a consensus about the clinically relevant cutoffs for the detection of minority genomes harboring one or more resistance mutations in HIV-1 quasispecies. These future achievements are intimately dependant on the growing sensitivity and reproducibility of HIV-1 genotyping technologies. Several assays based either on the recent developments of DNA sequencing (in particular, pyrosequencing and other platforms for ultra-deep parallel sequencing), on DNA microarray technology, or on novel nanotechnology-inspired biosensors are currently available for the characterization of minority genomes within HIV-1 and other viral quasispecies. Some of these ultrasensitive technologies could soon

be used to unveil the hidden memory of selected intrahost HIV-1 strains in the clinical setting; most likely, the minority report filled in the molecular biology laboratory will be of major importance for the management of the infected patient.

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