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Foot-and-mouth disease virus evolution: exploring pathways towards virus extinction

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1. Introduction: concepts in RNA virus evolution

Foot-and-mouth disease virus (FMDV) is one of the prototypes of antigenically variable virus, reflected in seven serotypes (A, O, C, Asia 1, SAT1, SAT2, SAT3), and many subtypes and variants, too numerous to be amenable to any reasonable cataloguing at present. The diversity of antigenic types creates difficulties for prevention of FMD by vaccination since there is no predictable, reproducible and effective protection that can be afforded by a limited number of vaccine antigens against multiple variants cocirculating in different world areas, and sometimes even within the same geographical area (DOEL 2003; SUTMOLLER et al. 2003). Traditionally, vaccine manufacturers have known that it is necessary to tailor vaccine composition to match the antigenic properties of the circulating FMDV in much the same way as the influenza vaccines must be periodically updated. Chemically defined vaccines would be desirable but they will often fail to provide protection against the diverse and heterogeneous array of virus forms to be controlled. Vaccine inefficacy is a major challenge for prevention against diseases caused by variable RNA viruses such as AIDS, hepatitis C and possibly SARS, among many others.

Phenotypic variation of FMDV has been known since the early work of C.R. Pringle, H. Bachrach and others [reviewed in (BACHRACH 1968) and (DOMINGO et al. 2003)]. There is now little doubt that antigenic variation of FMDV is consequence of the error-prone replication of the viral genome, visualized as variability of antigenic epitopes. This feature is shared with other RNA viruses and it is due to the lack (or low efficiency) of proofreading-repair and postreplicative-repair activities during RNA-dependent RNA and DNA synthesis (HOLLAND et al.

1982; DRAKE and HOLLAND 1999; DOMINGO et al. 2001). Mutation rates during RNA replication and retrotranscription average 10^{-3} to 10^{-5} misincorporations per nucleotide copied. Together with homologous and nonhomologous recombination and genome segment reassortment (in viruses with segmented genomes), these mechanisms provide the molecular scenario on which virus diversification and adaptability are built [overviews in the different chapters of (MORSE 1993; MORSE 1994; GIBBS et al. 1995; DOMINGO et al. 1999; DOMINGO et al. 2001; DOMINGO 2003)]. Despite high error rates affecting all RNA viruses examined to date (DRAKE and HOLLAND 1999) the extent of antigenic variation and, therefore, the resulting problems of low vaccine efficacy derived from variation, do not affect equally all RNA viruses. For example, there is one serotype of Mengo virus, three of poliovirus, seven of FMDV and more than one hundred of human rhinoviruses, despite monoclonal-escape mutants arising with comparable frequencies in the range of 10^{-3} to 10^{-5} for these picornaviruses [values have been compared in (DOMINGO et al. 2002)]. This indicates the existence of constraints to antigenic diversification operating with different intensity, even among viruses sharing a pattern of genetic organization and features of their virion structure. Defining the molecular basis of such constraints remains a challenge for virology, that requires bridging biochemistry with evolutionary biology.

Being FMDV a picornavirus of about 8,300 nucleotides with a highly compact genetic information, a single open reading frame, *cis*-acting functions, regulatory regions dependent on precise spatial structures, several multifunctional proteins, and from all evidence replicating close to the error threshold for maintenance of genetic information (section 2), it is remarkable that a large repertoire of many

different mutations are tolerated by the virus. Mutations may affect important functions such as translation or replication efficiency, cell tropism or host range, and they have been produced during virus replication, without the need of chemical mutagenesis [specific cases reviewed in (BARANOWSKI et al. 2001; BARANOWSKI et al. 2003; JACKSON et al. 2003; MASON et al. 2003)]. FMDV mutants produced during replication in cell culture or in animals are under examination and provide valuable tools to help understanding FMDV biology.

Genetic variation of FMDV, as that of other viruses, results in an increasing amount of nucleotide and amino acid sequence information that demands an orderly treatment of data to arrive at a meaningful understanding of the origins of genetic diversity. Genomic sequences have been analyzed by phylogenetic and statistical procedures to define relationships among dominant sequences found in the isolates from infected animals. The result of such analyses is a new genotypic classification of FMDV that is gradually replacing the classical classification based on serology. Phylogenetic studies have also permitted to trace the origin of FMD outbreaks, contributing to the molecular epidemiology of this pathogen (see chapter……by……..). An understanding of the origins of genetic diversity is increasingly achieved by analyzing in detail the genetic population structure of the virus as it replicates in infected hosts, or in cell culture. Probing into the fine structure of viral genomes at the population level has been possible through the application of biological and molecular cloning, combined with rapid nucleotide sequencing.

The population size and genetic heterogeneity of viral populations are increasingly recognized as relevant to viral pathogenesis. Model studies on passage

of infectious clones under controlled environments and designed population regimes can provide insight into the major evolutionary forces acting on viruses. For viruses that can produce plaques on cell monolayers (or cause infection from a single viral particle by end-point dilution), the progeny of a single infectious genome can be analysed. In sequence screenings based on biological clones, there is a bias towards scoring genomes which are infectious in the particular cell line chosen, but not in other cells or in some animal hosts. Reverse transcription of genomic RNA and PCR amplification (RT-PCR), followed by molecular cloning and sequencing of individual clones, offers an alternative means to examine viral populations that does not depend on infectivity. Here a bias may come from low fidelity of the enzymes used for RT-PCR (that may result in an overestimate of nucleotide sequence heterogeneity), or from a limitation in the number of viral RNA template molecules amplified by RT-PCR (resulting in an underestimate of nucleotide sequence heterogeneity). Both potential biases can be easily avoided by appropriate control experiments [experimental details using FMDV have been given in Arias *et al.* (2001) and Airaksinen *et al.* (2003)].

Extensive studies with FMDV and other RNA viruses, involving the analysis of biological and molecular clones have documented that at the population level viral genomes in infected natural hosts and cell cultures, during acute and persistent infections, consist of complex mutant distributions termed viral quasispecies (EIGEN 1971; DOMINGO et al. 1978; EIGEN and SCHUSTER 1979; EIGEN and BIEBRICHER 1988; EIGEN 1996; DOMINGO et al. 2001). This means that the nucleotide sequence determined for a virus isolate (the average or consensus sequence of that particular isolate) often does not exist physically in the population, or it exists only as a minority subpopulation within a larger mutant

spectrum. The composition of an RNA virus is “statistically defined but individually indeterminate” (DOMINGO et al. 1978). This population structure is a consequence of the high input of mutations during replication, and underlies virus adaptability since viruses replicate essentially as pools of genetic and phenotypic variants. The individual components of the mutant spectrum are ranked according to relative fitness (a measure of relative replication capacity in a given environment), and most of them, when they replicate, display lower fitness than the average for the population from which they were isolated (DOMINGO et al. 1978; DUARTE et al. 1994). Fitness moreover, is unavoidable a property of ensembles of individuals since even virus from a single plaque is a mutant distribution (ESCARMÍS et al. 1996; ESCARMÍS et al. 2002). Subpopulations of viral genomes provide a reservoir of genomes ready to become the dominant subset in the face of an environmental challenge (e.g. an immune response, the presence of an inhibitor that targets the dominant genome class, or the encounter with a new host cell type, among others). The generation of quasispecies swarms is the first stage in the process of genetic diversification of viruses that occurs within infected hosts. Diversification is more clearly manifested upon host-to-host transmission when one or a few founder viruses from a mutant spectrum replicate in a different environment. Further diversification in nature is a complex process, poorly understood in molecular terms, and thought to be influenced by positive selection and random drift of genomes [reviewed in different chapters of (MORSE 1993; MORSE 1994; GIBBS et al. 1995)].

Several experiments have shown that virus evolution is directly relevant to the generation of disease or to disease progression within infected individuals. Classical examples are mutations that render attenuated poliovirus virulent, and progression to AIDS associated with HIV-1 evolution. In this respect, the elegant

work of (KIMATA et al. 1999) showed that simian immunodeficiency virus molecular clones synthesized from virus isolated from monkeys at different stages of disease, reproduced the disease stage of the parental monkey when inoculated into healthy monkeys. Furthermore, many cases of genetic change associated with a modification of host cell tropism and host range with implications in viral pathogenesis have been described [reviewed in (BARANOWSKI et al. 2003)]. Therefore, virus evolution is one of the determinants of viral disease. A common misunderstanding is the thought that many mutations are needed for a substantial biological modification such as an alteration of virus host range or virulence. This is not necessarily the case. One or a few mutations (with a good probability of being represented in mutant spectra of viral quasispecies) may suffice to alter virulence and other important biological traits of viruses. A case involving FMDV is the demonstration that one amino acid replacement selected during replication of a swine virus in guinea pigs determined the capacity to produce disease in the new host (NÚÑEZ et al. 2001). The problem of relating a genetic change with a phenotypic alteration does not arise commonly from the number of mutations but from the fact that several unrelated mutations or multiple unrelated combinations of few mutations may lead to similar phenotypic alterations.

Additional implications of quasispecies dynamics for RNA virus biology have been recently reviewed (DOMINGO et al. 2001; DOMINGO 2003) and they will not be further discussed here, except with respect to molecular mechanisms of virus extinction and recent trends in the development of new antiviral strategies, the central topics of this chapter.

2. Accumulation of deleterious mutations: rate, mode, mechanisms.

Several concepts of population genetics have been very useful for the understanding of quasispecies dynamics. One of them is the accumulation of deleterious mutations in asexual populations of organisms when no compensatory mechanisms such as recombination intervene, a process termed Muller's ratchet (MULLER 1964; MAYNARD-SMITH 1976). The operation of Muller's ratchet was first documented with an RNA virus by Lin Chao (CHAO 1990) working with phage ϕ6. These results were then extended to VSV (DUARTE et al. 1992), FMDV (ESCARMÍS et al. 1996) and HIV-1 (YUSTE et al. 1999). Experimentally, an increase in deleterious mutations and fitness loss can be demonstrated upon serial plaque-to-plaque transfers of virus, in which virus replication is limited to the development of a plaque on the cell monolayer (Fig. 1). Fitness loss associated with accumulation of deleterious mutations is in contrast with fitness gain upon large population passages of virus (NOVELLA et al. 1995a) (Fig. 2). In the latter situation, a competitive optimization of the mutant distributions occurs, resulting in selection of mutant distributions that show high fitness in the environment in which replication takes place. The initial fitness of the virus determines whether a given population size involved in replication will lead either to an increase or to a decrease in relative fitness (NOVELLA et al. 1995b; NOVELLA et al. 1999).

Nucleotide sequence comparisons carried out with FMDV have defined multiple molecular pathways for fitness loss (ESCARMÍS et al. 1996) or for fitness gain (ESCARMÍS et al. 1999). Remarkable differences were noted in the types of mutations accumulating in the FMDV genome in the course of plaque-to-plaque transfers and those observed among natural isolates, or laboratory populations evolved without bottlenecking. Specifically, in clones derived by serial plaque transfers, 50% of the amino acid substitutions affecting the viral capsid were

located at internal capsid sites, which are highly restricted for variation during natural evolution or large population passages of FMDV (ACHARYA et al. 1989; MATEU et al. 1994). In the course of at least 200 plaque-to-plaque transfers of several viral clones, point mutations accumulated in a nearly linear fashion at a rate of about 0.3 mutations per genome per transfer. This is an average rate of accumulation observed in the consensus sequence of the virus population present in a single plaque, but it does not mean that three plaque transfers are needed for one mutation to occur. During each plaque development multiple mutations occur (as expected from high mutation rates; section 1) and virus from individual plaques is genetically heterogeneous (ESCARMÍS et al. 1996; ESCARMÍS et al. 2002). Mutations that accumulated upon plaque transfers of FMDV were clustered at some genomic loci (different for independent lineages) rather than being randomly distributed along the genome. In clusters, the mutation frequencies were four-to five-fold larger than the average for the entire genome, and the difference with the average mutation frequency for the entire genome was statistically significant. The basis for such mutation clustering is not well understood (ESCARMÍS et al. 2002). Possible interpretations include a reduced copying fidelity of the viral replicase at genomic regions affected by template structure or preexisting mutations, or the occurrence of compensatory mutations in the proximity of those that are deleterious. In addition, many clonal lineages acquired an elongation of four adenylate residues that precede the second AUG translation initiation codon, which created an internal oligoadenylate, a genetic lesion that had never been observed in FMDV. The oligoadenylate was variable in length, heterogeneous within some viral plaques, and associated with fitness loss of FMDV (ESCARMÍS et al. 1996; ESCARMÍS et al. 1999). It is one of the genetic markers used to demonstrate the

presence of a molecular memory in viral quasispecies (RUÍZ-JARABO et al. 2000; ARIAS et al. 2001; RUÍZ-JARABO et al. 2002). The oligoadenylate permitted the experimental demonstration of multiple, alternative pathways for fitness recovery when the debilitated clones were subjected to large population passages. Alternative pathways observed were the shortening of the elongated oligoadenylate that on occasions yielded the wild type sequence (true reversion), and a deletion of 69 residues spanning the site of the polyadenylate extension (ESCARMÍS et al. 1999). Interestingly, this deletion resulted in viable viruses whose genomes included only twelve nucleotides between the two AUG translation initiation codons (Fig. 3). The mechanism proposed for the generation and elongation or shortening of the internal oligoadenylate tract is polymerase slippage that leads to misalignment mutagenesis (RIPLEY 1990; ARIAS et al. 2001). These results with FMDV emphasize how the dynamics of viral infections (e.g. occurrence and severity of genetic bottlenecks, intervention of large population infections, etc.) can profoundly affect the types and numbers of mutations observed in a virus genome [the portion of "sequence space" the mutant spectra occupy (EIGEN and BIEBRICHER 1988)] despite using the same replication machinery with its copying fidelity properties, within the same host cells.

3. Resistance to extinction despite accumulation of mutations: observations and modeling.

Despite a nearly linear accumulation of mutations in FMDV clones subjected to plaque-to-plaque transfers, the virus showed a remarkable resistance to extinction. An FMDV population (either obtained from a plaque or from an infection in liquid culture medium) is considered extinct when upon at least three blind

passages in cell culture under optimal infection conditions, no infectivity and no FMDV-specific RT-PCR-amplifiable material can be recovered. The amount of infectious virus found in individual plaques developed during a given time period in the same environmental conditions, was taken as an approximate measure of relative fitness (ESCARMÍS et al. 2002). Fitness decrease upon serial plaque-to-plaque transfers of several FMDV clones was biphasic. An initial phase of exponential decrease was followed by a second phase in which fitness values displayed large fluctuations around an average constant value; the amplitude of the fluctuations tended to be larger the lower the fitness values (ESCARMÍS et al. 2002; LÁZARO et al. 2002) (Fig. 4).

A numerical model was developed that provided clues to a molecular interpretation of the experimental results. A critical feature of the model is the occurrence with low probability of advantageous mutations that permit reaching an equilibrium between the trend to eliminate individuals that have attained very low fitness values and the probability of selecting for the subsequent transfer individuals with compensatory mutations. As a result of both processes a stationary state of constant average fitness is achieved (LÁZARO et al. 2002). Nucleotide sequencing of genomic RNA from clones of successive plaque transfers indicated that mutations were associated with fitness fluctuations. In particular, in clones with the internal oligoadenylate, a shortening of the homopolymeric tract, or its interruption by A → G transitions, resulted in fitness increase (ESCARMÍS et al. 2002). The relative fitness was inversely related to the length of the oligoadenylate tract (Fig. 5). Because of these mutation-associated fluctuations in viral production, the FMDV clones displayed a remarkable resistance to extinction. Here a constant mutational input serves to attenuate the effects of Muller's ratchet to render them

unnoticeable regarding virus survival. Manrubia and associates (MANRUBIA et al. 2003) have explored a mean-field theory that in fact represents an extension of the model developed for FMDV to any exponentially growing population of mutating replicons. The mean field theory proposed permits an exact formal analysis of various dynamical regimes. When the population is subjected to strong bottlenecks, large fluctuations of viral yield at each passage are expected, but they do not drive the population to extinction. A precise mathematical form describes the fluctuations as observed experimentally. When the population is allowed to grow exponentially for many replication cycles, the relative proportion of different mutant types attains fixed values. Remarkably and unexpectedly, in this model, for high enough mutation rates, the most represented variant is not the one with the highest fitness (MANRUBIA et al. 2003).

In a further theoretical development linked to the results on repeated bottlenecking of FMDV, Lázaro et al. (2003) have shown that the fluctuating pattern of fitness values of FMDV clones follows a Weibull distribution. This is a type of statistical distribution (WEIBULL 1951) that describes unrelated processes such as fragmentation of materials, cardiac contractions, and time between events of the disease paroxysmal atrial fibrillations (ARAKI et al. 1999; ROSE et al. 1999). This pattern essentially reflects the large variations in the initial fitness of the founder virus at each bottleneck event, which are exponentially enhanced during plaque development. Variations in fitness have their origin in the mutations occurring in the viral genome during plaque development, and result from the extreme complexity of the host-virus interaction, involving multiple viral and host cell functions (DOMINGO 2003; MANRUBIA et al. 2003). As the virus becomes more debilitated by deleterious mutations, compensatory mutations play a more

relevant role to increase the fitness of the virus, contributing in this way to the fluctuating pattern of fitness values and survival of genome subsets from the population (LÁZARO et al. 2002; MANRUBIA et al. 2003).

Concepts that find application to virology may arise as an integration of results from experimental virology with population genetics and physics. One such concept is complexity which refers to systems made of multiple elements, whose properties (or behaviour) cannot be anticipated merely as the sum of contributions of the individual elements that comprise the system (GELL-MANN 1994). FMDV, as other RNA viruses, has a compact genetic information in its genome (section 1), so that many genomic regions are involved in multiple functions. Therefore mutations, so frequent in each replication round, when accumulated as the result of bottlenecks, may trigger a cascade of perturbations that lead to large variations in the fitness of the individual components of a lytic plaque. That this is so (versus the alternative of accumulation of mutations that sum small biological effects) has been unveiled by the numerical analyses of plaque-to-plaque virus yield values that have shown that the system follows a Weibull distribution (MANRUBIA et al. 2003). The effect of mutations, however complex and continuous, did not drive the entire FMDV population to extinction. Alternative pathways of accumulation of mutations that lead to effective extinction of viruses must be sought.

4. Lethal mutagenesis or the transition into error catastrophe: a pathway towards virus extinction.

A consequence of error-prone replication and quasispecies dynamics is the existence of an error threshold for maintenance of genetic information. This important concept was proposed on a theoretical basis (SWETINA and SCHUSTER

1982; EIGEN and BIEBRICHER 1988; NOWAK and SCHUSTER 1989; ALVES and FONTANARI 1998; NILSSON and SNOAD 2000; EIGEN 2002), and has been supported by an increasing number of experimental results with different viruses (HOLLAND et al. 1990; LOEB et al. 1999; CROTTY et al. 2000; LOEB and MULLINS 2000; CROTTY et al. 2001; LANFORD et al. 2001; GRANDE-PÉREZ et al. 2002; DOMINGO et al. 2003; SEVERSON et al. 2003). The first experimental evidence of a transition of virus into error catastrophe was obtained by J.J. Holland and his colleagues who documented the adverse effect of chemical mutagenesis on VSV and poliovirus (PV) (HOLLAND et al. 1990; LEE et al. 1997). These results suggested that VSV and PV replicate close to the error threshold. Increases in mutation rate should result in violation of the error threshold and an irreversible transition from a productive viral infection into an abortive viral infection, reflected in virus extinction. It must be emphasized that virus entry into error catastrophe implies virus extinction, not merely a decrease in virus titer.

Experiments with FMDV have been designed to evaluate the effect of relative viral fitness and viral load (the number of infectious particles that participates in the infection) in virus extinction by mutagenic base analogs. For a given viral load, a low relative fitness favors virus extinction (SIERRA et al. 2000; PARIENTE et al. 2001). This satisfies a prediction of quasispecies theory since a lower viral fitness implies a lower superiority of the master sequence in the quasispecies distribution, and this is one of the parameters that determine complexity of the genetic information that can be maintained (the position of the error threshold) (SWETINA and SCHUSTER 1982; NOWAK and SCHUSTER 1989; ALVES and FONTANARI 1998; NILSSON and SNOAD 2000; EIGEN 2002). For a given relative fitness, the lower the viral load, the more likely it was to drive FMDV to extinction by a given

dose of mutagenic agent (SIERRA et al. 2000; PARIENTE et al. 2001). These observations led to investigations of FMDV extinction by the combined action of the mutagenic base analog 5-fluorouracil (FU) and the antiviral inhibitors guanidine hydrochloride and heparin. Some of these treatments led to systematic extinction (47 times out of 47 attempts) of high fitness FMDV in less than five passages in cell culture (PARIENTE et al. 2001). Interestingly, extinction of high fitness FMDV was achieved in combinations that included FU but not with guanidine hydrochloride and heparin alone, even when these two inhibitors exerted the same inhibitory effect as FU in one passage (PARIENTE et al. 2003). These results suggest that combinations of viral-specific mutagenic agents and antiviral inhibitors could provide suitable treatments to eradicate virus from infected organisms.

The FMDV model provided also an experimental system to approach the problem of virus escape due to resistance to antiviral inhibitors, an evolutionary phenomenon that greatly impairs treatment by antiviral inhibitors of important diseases such as AIDS, hepatitis C or influenza (RICHMAN 1996; DOMINGO et al. 2001; MENÉNDEZ-ARIAS 2002b). A detailed analysis of the FMDV populations that escaped extinction showed that in all cases mutations that confer resistance to guanidine hydrochloride and/or heparin were selected (PARIENTE et al. 2003). In contrast, the consensus sequence of preextinction populations (those preceding extinction) did not show any mutation relative to the wild type. In the process towards extinction by a combination of FU and guanidine hydrochloride, there was a 1000-fold reduction in virus titer, and a 100-fold reduction in the amount of viral RNA (PARIENTE 2003). This and other observations (C. González-López and N. Pariente, unpublished results) suggest that there is a considerable amount of non-infectious, mutagenized RNA in the process of transition to error catastrophe

(PARIENTE et al. 2003). These results reinforce the conclusions that mutagenesis rather than inhibition of viral-specific RNA synthesis is the main mechanism of action of FU in the extinction of FMDV. Similar conclusions have been reached in studies with the prototype arenavirus lymphocytic choriomeningitis virus (LCMV) (GRANDE-PÉREZ et al. 2002; DOMINGO et al. 2003). Intracellular UTP pools were depleted in FU-treated cells while there was an accumulation of fluorouridine triphosphate (FUTP), resulting in approximately 12 times more intracellular FUTP than UTP. The other NTPs remained close to normal levels (PARIENTE et al. 2003). These measurements strongly suggest that FU-mediated mutagenesis of FMDV is associated with incorporation of fluorouridine monophosphate into viral RNA rather than with nucleotide pool imbalances. Experiments are in progress to further investigate this point.

The transition into error catastrophe was accompanied by increases in mutant spectrum complexity as measured by mutation frequencies (proportion of mutated positions) and Shannon entropies (proportion of non-identical genomes). Such increases varied depending on the virus (poliovirus, HIV-1, FMDV or LCMV), the genomic region analyzed, and the mutagenic treatment (LOEB et al. 1999; SIERRA et al. 2000; CROTTY et al. 2001; PARIENTE et al. 2001; GRANDE-PÉREZ et al. 2002). In the case of FMDV, mutation frequencies in the mutant spectra of populations subjected to FU mutagenesis were 1.5- to 6-fold larger than for control populations subjected to parallel passages in the absence of mutagen. The maximum relative increases in complexity were seen in the polymerase (3D)-coding region, a genomic region which, under unperturbed conditions of replication, is highly conserved, and that usually displays a very narrow mutant spectrum (SIERRA et al. 2000). Despite increases in mutant spectrum complexity, consensus

sequences in preextinction FMDV populations did not vary with respect to the parental virus, suggesting that mutagenic pressure impairs adaptive selection of mutant distributions with new dominant sequences. In contrast, genomic consensus sequences of FMDV populations that were subjected to serial passages in the presence of mutagenic agents, but that did not result in virus extinction, showed mutations in the consensus sequence with respect to the parental virus (SIERRA et al. 2000). This result suggests that in this case adaptive mutations occurred that contributed to elude extinction, as also observed with extinction-escape mutants of FMDV harboring inhibitor-resistant mutations when mutagenesis was insufficient to achieve virus extinction (PARIENTE et al. 2003).

Ribavirin (1-β-D-ribofuranosyl-1, 2, 3-triazole-3-carboxamide) is a broad-spectrum nucleoside analog that can exert its antiviral activity through several mechanisms (SNELL 2001; ZHANG et al. 2003b). Interestingly, this licensed drug has been shown to be mutagenic for a number of RNA viruses (CROTTY et al. 2000; CROTTY et al. 2001; LANFORD et al. 2001; MAAG et al. 2001; AIRAKSINEN et al. 2003; SEVERSON et al. 2003). Ribavirin triphosphate can be incorporated by RNA-dependent RNA polymerases (CROTTY et al. 2000; CROTTY et al. 2001; MAAG et al. 2001) thus providing a molecular interpretation of its mutagenic action. Recently, a poliovirus polymerase mutant with decreased sensitivity to ribavirin has been characterized (PFEIFFER and KIRKEGAARD 2003) emphasizing the need to cross the error threshold to avoid selection not only of virus mutants resistant to inhibitors that may be used in combination with mutagens (AIRAKSINEN et al. 2003), but also of mutants that may show decreased sensitivity to mutagenic agents. However, mutagenesis is not the only mechanism by which ribavirin exerts its antiviral activity. Its potent anti-arenavirus activity

was not associated with significant increases in mutant spectrum complexity of LCMV (DOMINGO et al. 2003), a result which is in contrast with the effects of FU in the same system (GRANDE-PÉREZ et al. 2002; DOMINGO et al. 2003). Application of microarray-based detection of perturbations in cellular gene expression documented multiple alterations of gene expression that were associated with administration of ribavirin in cells infected with respiratory syncytial virus (ZHANG et al. 2003b).

Ribavirin can eliminate FMDV from persistently infected cell cultures (DE LA TORRE et al. 1987). To examine whether this curing activity was associated with enhanced mutagenesis, the alterations of mutant spectra complexity of persistent FMDV as a result of treatment with ribavirin or with mycophenolic acid were compared (AIRAKSINEN et al. 2003). Both drugs are inhibitors of inosine monophosphate dehydrogenase (IMPDH), a key enzyme of the pathway of the synthesis of GTP, and therefore, administration of either of these two drugs results in similar intracellular nucleotide pool imbalances. The critical difference between the two inhibitors is that ribavirin triphosphate (RTP) can be a substrate for viral polymerases while mycophenolic acid cannot be incorporated into polynucleotide chains. Mycophenolic acid-induced mutagenesis of FMDV was weak and reversed by addition of guanosine to the culture medium and, thus, it was probably due to nucleotide pool alterations. In contrast, ribavirin addition resulted in higher mutation frequencies (AIRAKSINEN et al. 2003) which reached levels comparable to those observed in other viral systems (CROTTY et al. 2000; CROTTY et al. 2001; CONTRERAS et al. 2002; SEVERSON et al. 2003; ZHOU et al. 2003). The antiviral effect of ribavirin was diminished by guanosine addition while its mutagenic activity was not. These observations suggest that ribavirin-mediated curing of

FMDV from persistently infected cultures was associated with at least two effects: mutagenesis and inhibition of IMPDH (AIRAKSINEN et al. 2003). Despite ribavirin having multiple mechanisms of action, the demonstration that at least in some cases its antiviral activity may be associated with a mutagenic activity has represented an important progress in the prospects of developing error catastrophe as a new antiviral strategy (GRACI and CAMERON 2002).

De la Torre and colleagues (DOMINGO et al. 2003) have shown that treatment of mice with FU prevented the establishment of a persistent infection with LCMV. This important result constitutes a proof of principle of the feasibility of a mutagenesis-based, antiviral approach *in vivo*.

5. Advantages and limitations of error catastrophe as an antiviral strategy

A comparison of the results with FMDV showing the robust resistance of this virus to extinction when mutations accumulate during bottleneck transfers (ESCARMÍS et al. 2002; LÁZARO et al. 2002; MANRUBIA et al. 2003), with results showing extinction by mutagenic treatments (SIERRA et al. 2000; PARIENTE et al. 2001; AIRAKSINEN et al. 2003; PARIENTE 2003) points to modulation of the mutagenic input as critical for virus survival. This does not mean that upon serial bottleneck transfers extinction events are rare. They are probably frequent, but the mutant repertoire allows for a viable virus (in this case "viable" means "able to form a plaque") to arise. An excessive mutation rate suppresses this "viability-escape" potential of the mutant reservoir. The critical contribution of mutation rates to extinction, predicted by theoretical treatments (SWETINA and SCHUSTER 1982; EIGEN and BIEBRICHER 1988; NOWAK and SCHUSTER 1989; ALVES and FONTANARI 1998; NILSSON and SNOAD 2000) has been documented experimentally with the comparative studies with FMDV (section 3 and 4). The irreversibility of the transition provides a definitive advantage of error catastrophe versus inhibition as an antiviral design [(AIRAKSINEN et al. 2003); C. González-López *et al*, manuscript in preparation].

Despite the promising developments summarized in section 4, a clinical application of lethal mutagenesis requires addressing a number of issues: (i) The specificity for viral replicases and retrotranscriptases of the antiviral mutagens to be used alone or in combination with antiviral inhibitors. This is a key point that requires the development and testing of new mutagenic agents that can be incorporated by viral enzymes but not by cellular enzymes. (It must be said,

however, that many drugs that have been licensed as antiviral agents show considerable toxicity for cells and organisms). (ii) A second problem to be addressed is that the concentration of mutagenic agent(s) at the sites of viral replication must be sufficiently high to provoke virus replication to cross the error threshold. Insufficient mutagenesis could favor survival of virus mutants with unpredictable biological properties, or even mutants manifesting resistance to mutagenic agents (PFEIFFER and KIRKEGAARD 2003). (iii) To limit possible resistance to lethal mutagenesis it may be necessary to use simultaneously more than one mutagen to avoid viral escape (as with combination therapy with inhibitors). (iv) In the case of retroviruses, the integrated provirus will be largely immune to mutagenic treatments since proviral DNA is copied by the cellular replication machinery as if it were a cellular gene. Retrovirus entry into error catastrophe may necessitate combining activation of provirus to enter the particle formation pathway, together with mutagenic treatments.

These limitations for an eventual clinical application of error catastrophe are not substantially different than those limitations often encountered with current antimicrobial treatments, and they should encourage rather than discourage research on antiviral treatments aimed at virus extinction through enhanced mutagenesis. This new line of research is favored by an increasing understanding of the molecular basis of polymerase copying fidelity (MENÉNDEZ-ARIAS 2002a), as well as by expanding possibilities for the design of new mutagenic base analogs, useful also for other types of medical interventions such as cancer therapy. When low toxicity and means to reach effective mutagen or mutagen-inhibitor combinations are achieved, chances of success will be high. This great potential will

only be realized as a result of gradual accumulation of basic information using multiple virus-host systems.

6. Conclusions and prospects

In this article we have summarized experimental and theoretical approaches to the understanding of quasispecies dynamics of FMDV aimed at defining parameters that can shift virus populations from sustained survival to extinction. One of the advantages of exploiting evolutionary concepts to design antiviral strategies is that developments can benefit from studies with multiple virus-host systems since the molecular bases of the designs are shared by all viral systems. One of the common threads that underlie quasispecies dynamics —on which error catastrophe methodology is constructed— is error-prone replication. Low fidelity of template copying is expected to occur whenever RNA is the genetic material (in all riboviruses and retroviruses) or RNA is a replicative intermediate in DNA viruses (in hepadnaviruses such as hepatitis B virus). Even if some proofreading activity operated during replication of the largest RNA genomes —for example, an activity of the type described for human influenza virus polymerase (ISHIHAMA et al. 1986)— it is unlikely that post-replicative correction pathways would be efficient on those RNA genomes. Therefore, high mutation rates, population heterogeneity, quasispecies dynamics and the potential for rapid evolution in nature stand as general features of RNA viruses and probably also of some DNA viruses (DOMINGO 2003). These general features encourage equally general antiviral designs such as lethal mutagenesis.

There are precedents in biological systems of exploitation of enhanced mutagenesis as a defense mechanism of cells against invading molecular parasites.

Filamentous fungi such as *Neurospora crassa* have evolved a mechanism against DNA with repeated nucleotide sequences that can penetrate into their cells, consisting in producing mutations at each of the repeat copies. This mechanism is known as repeated-induced point mutations (RIP) [(KINSEY et al. 1994); reviews in (BUSHMAN 2002) and in (ARNOLD and HILTON 2003)]. The existence of an innate cellular immunity to retroviral infections has recently been described (HARRIS et al. 2003; LECOSSIER et al. 2003; MANGEAT et al. 2003; ZHANG et al. 2003a). This cellular defense is mediated by mutagenesis of the viral genome through cytidine deamination which greatly impairs expansion of retroviruses. Protein vif of HIV-1 overcomes this mutagenesis effect through a still unknown mechanism. Therefore, a mutagenesis-based antiviral approach to drive virus to extinction may not be foreign to the natural mechanisms that have permitted survival of organisms in the face of perturbing molecular parasites.

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Figure legends

Figure 1. A scheme of a viral quasispecies and the accumulation of mutations upon plaque-to-plaque transfers. A typical RNA virus mutant distribution is shown on the right; genomes are depicted as horizontal lines and mutations are represented by different symbols on the lines. Plaque-to-plaque transfers (small arrows) force the population through successive populations with a modified consensus sequence (bottom lines). Note that the consensus sequences are not represented in their respective mutant spectra. Since deleterious mutations tend to be more frequent that neutral and advantageous mutations, relative fitness (upper arrow) tends to decrease initially. When fitness is low the dynamics of fitness variation becomes very complex (see text).

Figure 2. A scheme of a viral quasispecies and adaptive evolution upon large population passage of the viral population. Symbols to depict viral quasispecies are as in Figure 1. Large population passages (thick central arrows) result in optimization of the mutant distribution and adaptation to the environment in which replication takes place, seen as a change in the consensus (or average sequence) of the population. In this case relative fitness increases (upper arrow). When fitness reaches high values, the population size may become a limiting factor for further increases of fitness, and the population dynamics becomes very complex (see text).

Figure 3. Position of a 69 nucleotides deletion (Δ 69) produced upon large population passages of an FMDV clone containing an internal oligoadenylate tract. Δ 69 is located between the two functional AUG protein synthesis initiation codons. The internal oligoadenylate (An) precedes the second functional AUG. Based in results reported in (ESCARMÍS et al. 1996; ESCARMÍS et al. 1999).

Figure 4. Evolution of relative fitness of an FMDV clone subjected to plaque-to-plaque transfers. At each plating on a BHK-21 cell monolayers, a plaque (developed for 24 hours under a semisolid agar overlay) was chosen at random and the infectious virus present in it was determined by a plaque assay (values in ordinate). Virus from the same plaque was plated again and the process repeated a total of 125 times (abcissa). A biphasic evolution and large fluctuations of fitness values can be observed. Similar findings were obtained with additional FMDV clones. Based in results reported and discussed in (ESCARMÍS et al. 2002; LÁZARO et al. 2002; MANRUBIA et al. 2003).

Figure 5. Length of the internal oligoadenylate tract and infectivity of FMDV clones. The genomic region spanning the internal oligoadenylate tract (compare with Fig. 3) was amplified by RT-PCR in the presence of S^{35}-dATP. The amplified products (a 198 bp amplicon) was analyzed by electrophoresis through a 6% polyacrylamide sequence gel (top part of figure). The number of PFU contained in the corresponding plaques was also determined (bottom part of figure). This and other results documented that a longer oligoadenylate implied a lower infectivity. Based in experiments reported in (ESCARMÍS et al. 2002).