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Frequency-dependent selection in human immunodeficiency virus type 1

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Genetic variation is the main evolutionary strategy adopted by RNA viruses and retroviruses. Evolution operates through competition between different individuals in the same environment, resulting in the imposition of the fittest variant. The process of competition could be affected by various factors, including the frequency of the different competing individuals. In order to investigate this aspect, individual virus populations derived from a human immunodeficiency virus type 1 isolate were studied at different competing proportions. The dynamics of variant imposition in each competition experiment permitted the detection of frequency-dependent selection (FDS); i.e. the imposition of variants is related to their biological fitness, which is also affected by the proportions at which they compete. The existence of FDS in different viruses with RNA genomes would indicate a general mechanism favouring genetic heterogeneity.

Genetic variability is a key property of living organisms, although its extent varies in different species and individuals. Because of the high genetic variation, short duplication times and large population sizes, RNA viruses and retroviruses have become extremely interesting for evolutionary studies (Domingo *et al.*, 1985, 1996; Domingo & Holland, 1997; Moya *et al.*, 2000). In human immunodeficiency virus type 1 (HIV-1), one of the most important human pathogens of the family *Retroviridae*, important evolutionary aspects have been addressed, such as the Muller's ratchet effect (Yuste *et al.*, 1999) and the role of virus fitness in the evolution of antiviral agent-resistant variants *in vivo* (Goudsmit *et al.*, 1997) and *in vitro* (Harrigan *et al.*, 1998).

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Virus populations are submitted to positive and negative forces that contribute either to the generation and accumulation of variation or to the homogenization of the virus population (Moya *et al.*, 2000). Among the non-viral factors implicated in virus evolution, frequency-dependent selection (FDS) has recently been detected as operating in the evolution of vesicular stomatitis virus (Elena *et al.*, 1997). In order to study this concept in a retrovirus, we have carried out experiments with HIV-1 by performing competition cultures among 10 biological clones derived from a Spanish HIV-1 isolate at three different competing proportions. In this experimental setting, considering all clones as different samples from the same virus population, we have detected FDS in the HIV-1 clones.

In a previous experiment on fitness decline in HIV-1 after bottleneck transmission, the biological fitness of 10 virus clones was determined. Initial isolate s61 was recovered by standard co-culture techniques and grown in MT4 cells (Sanchez-Palomino *et al.*, 1993). From this virus population, 10 biological clones were picked randomly in an MT4 plaque assay (Harada *et al.*, 1985) and were designated populations A1 to K1.

Quantification of the fitness of the 10 HIV-1 clones was performed in competition experiments with a genetically marked clone (J1) as described previously (Holland *et al.*, 1991). These competitions were performed at three different proportions, 1:9, 1:1 and 9:1, of each clone with J1 in 5×10^4 MT4 cells infected at an m.o.i. of 0·1 p.f.u. per cell over five passages. Virus was recovered in each passage from the supernatant when cytopathic effect was evident, and was used to infect fresh MT4 cells. Quantification of each of the competing virus populations was carried out by the heteroduplex tracking assay (HTA) (Delwart *et al.*, 1994). HTA was performed with proviral DNAs amplified from competition cultures by PCR in the V1–V2 region of the *env* gene, as described previously (Yuste *et al.*, 1999). The proportion of each molecular species in the competition cultures was determined by using a clone J1 probe labelled with [α-^{32}P]-dCTP. Samples were denatured at 94 °C for 2 min and then cooled rapidly (Delwart *et al.*, 1994). Heteroduplexes were

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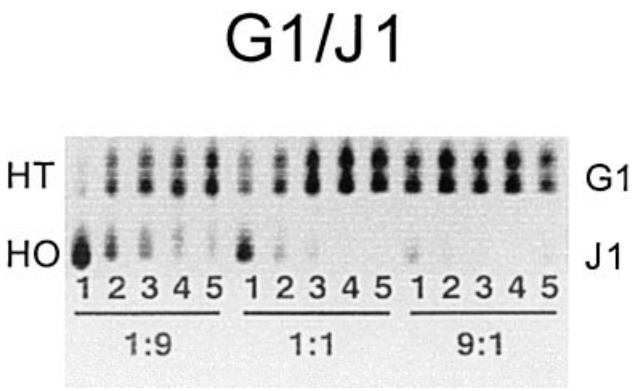

		1	2	3	4	5	
1:9	HT	91046	226439	281589	281683	294093	G1
	HO	339097	170005	139062	93989	65501	J1
1:1	HT	189195	238326	322364	353025	347462	G1
	HO	216311	71843	40863	17040	11764	J1
9:1	HT	305536	344283	310817	367253	309166	G1
	HO	75038	37288	26824	6167	0	J1

Fig. 1. HTA analysis of G1/J1 competition showing the five cultures of each of the three competing proportions, 1:9, 1:1 and 9:1. The proportion of the marked clone J1 was deduced from quantification of the homoduplex band (HO) and that of the competing virus G1 from the heteroduplex bands (HT). Bands were quantified by densitometry in a Fuji 2000 apparatus with the help of the PCBAS program. Arbitrary densitometric values deduced are represented in the lower panel. Each number corresponds to the quantification of the HTA bands displayed in the upper panel.

resolved in denaturing 15% polyacrylamide–8% urea gels and exposed in a Fuji 2000 densitometer for 2 h (Yuste *et al.*, 1999). An example of this competition quantification for G1/J1 viruses is shown in Fig. 1. During competition cultures, due to the quasispecies nature of RNA viruses and HIV-1, new variants were observed in some of the competition cultures in the V1–V2 region analysed. These variants interfered with heteroduplex quantification and, for this reason, these competitions were not considered. Some of these infections were repeated, such as the A1/J1, B1/J1 and K1/J1 competitions.

The fitness determinations were carried out in a set of 31 competition cultures at different proportions. In the present study, we used these cultures for frequency-dependent evolution calculations. The experimental ratios of each clone to the internal standard (J1) were obtained by densitometry of the HTA bands at each passage (see Fig. 1). All the clones analysed in the present study were derived from the s61 virus, and they could be considered to be random samples of the same virus population. Under such an assumption, each initial ratio, 1:9, 1:1 and 9:1, was repeated up to 12 times and the frequency of the competing clones was determined in five consecutive passages.

FDS was studied by two different procedures. The first consists of plotting the log-transformed initial and final ratios [log(noJ1/J1)] of the competing clones (Ayala, 1971; Ayala & Campbell, 1974). Under the null hypothesis of no FDS, we expected a regression line with a slope of 1. The stability (i.e. equilibrium) of the system can be analysed by studying the possible point of intersection between the estimated regression line and the line of slope 1, i.e. the frequency at which the two viruses have identical fitness. The equilibrium point is stable if, and only if, the slope of the regression line at the intercept is less than 1, i.e. if the relative fitnesses of both viruses are inversely related to their frequencies at that point. The study was performed by analysis of the slope obtained in each of the five passages from the different competitions.

The second method is a regression of the log-transformed ratio (noJ1/J1) versus number of passages ($t = 1$–5). If there is negative FDS, the population will converge at a given stable point. Additionally, the slopes of the regression lines were compared by co-variance analysis.

Table 1 summarizes the regression analyses carried out

Table 1. FDS analyses at different passages

Passage	a	$b \pm s_b$	d.f.	T (P)	Equilibrium point*	Ratio†
1	−0·28	0·70 ± 0·08	27	3·75 (< 0·05)	−0·93	0·39
2	1·00	0·66 ± 0·09	27	3·78 (< 0·05)	2·94	18·94
3	1·49	0·45 ± 0·18	17	3·04 (< 0·05)	2·71	15·02
4	1·77	0·62 ± 0·15	12	2·53 (< 0·05)	4·66	105·41
5	1·63	0·60 ± 0·24	9	1·68 (n.s.)	4·08	58·85

*Equilibrium is the point at which the output ratios intersect the slope $b = 1$ and is given by $a/(1-b)$.
†The ratio noJ1/J1 at equilibrium is obtained from $\exp(a/(1-b))$.
n.s., Not significant; d.f., degrees of freedom.

Table 2. Slope comparison by a co-variance analysis

The three types of culture at initial ratios of 1:9, 1:1 and 9:1 followed through five consecutive passages were analysed.

Source of variation	d.f.	Sum of squares	Mean square	F
Among slopes	2	0·0126	0·0063	0·006*
Deviations	96	100·2159	1·0439	–

*Not significant.

with all competitions in the five passages at the three different proportions. Except for passage 5, where there was no significant departure from a slope of $b = 1$, each passage showed statistical evidence of negative FDS (i.e. low frequency means high fitness) and equilibrium points. Due to the high heterogeneity among the replicates, as well as the reduced number of experimental points at passage 5, the estimated slope ($b = 0{\cdot}60$) probably also reflects negative frequency dependence. One other point requires some attention: except for passage 1, where the equilibrium point was placed at a value within the initial ratios of the study, the rest of the equilibrium points corresponded to ratios of a high concentration of non-J1 clones.

Table 2 shows the results of a co-variance analysis in which, on the one hand, regression of the log-transformed ratio noJ1/J1 to passage (1–5) was determined in three competitions with different initial ratios (1:9, 1:1, 9:1) while, on the other hand, the slopes obtained were compared. Although the slopes were not statistically different, it is worth noticing that the fitness function decreased in the three cases when the frequency of the non-J1 clone increased, as expected if negative FDS is present. The slopes for the 1:9, 1:1 and 9:1 initial ratios were respectively 0·5348, 0·5298 and 0·5128. The three regression lines intersected at passage 110, with a ratio that corresponds to an extremely high concentration of non-J1 clones, as obtained previously (see Table 1).

HIV-1 evolution *in vivo* and *in vitro* results from the interaction of different selective forces, both positive and negative, as well as other non-selective forces including genetic drift and sampling events. These factors result in the establishment of a swarm of variants, which has been termed a virus quasispecies (Domingo *et al.*, 1996; Moya *et al.*, 2000). Models have been proposed in which, assuming an infinite population, no bottleneck passages and population sizes much larger than the inverse of the mutation rate, the frequency of a mutation in a population can be estimated to be a function of the frequency of its occurrence in one replication cycle, and the fitness value of each clone corresponds to their representation in the quasispecies (Coffin, 1995). This model assumed a constant value of fitness. However, there are other examples in which the presence of a variant in a quasispecies is not directly linked to its fitness (de la Torre & Holland, 1990).

The experiments described here indicate that the fitness values of HIV-1 can vary, depending on the competition conditions and on the relative genetic composition of the population, which is evolving constantly due to virus replication. The results of this research, together with the description of FDS in vesicular stomatitis virus (Elena *et al.*, 1997), may indicate that this phenomenon is a general occurrence in the evolution of RNA virus populations. What could be the biological consequences of this effect? One is that the fitness of the virus is not constant, but varies depending on the genetic composition of the population; i.e. on the representation of the variants in the quasispecies. In consequence, if the fitness of a variant increases when the representation of the variant in the virus population decreases, the mechanism of FDS will result in the preservation of minor variants. As we may observe, this only happens when the minor variants have a higher fitness than higher-frequency variants. This observation of FDS has been suggested previously in a patient infected with HIV-1 (Holmes *et al.*, 1992). In the latter study, this phenomenon was observed by the antigenic evolution of V3 loop sequences. The authors observed that, in general, the most frequent sequence of each year was the one that suffered the greatest reduction in frequency in later samples, probably due to neutralization (Holmes *et al.*, 1992). In our study, FDS was observed in the absence of immune constraints. The observation of FDS in two different experimental settings, in the presence and absence of immune pressure, could support the generality of this concept in HIV-1.

It is a matter of speculation to decide which biological factors are responsible for fitness variation. Frequency dependence probably appeared as a consequence of differences among the members of the virus quasispecies with distinct replication efficiencies associated with several factors, including different relative rates of reverse transcription, translation or virus encapsidation and morphogenesis, as well as interactions between virus clones. In summary, this study confirms that FDS operates in the evolution of different RNA viruses. FDS could represent a general mechanism that contributes to the maintenance of virus quasispecies complexity by favouring minor variants.

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