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Infection, Genetics and Evolution 1 (2001) 41–48

Infection, Genetics and Evolution

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Transmission bottlenecks and the evolution of fitness in rapidly evolving RNA viruses

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Received 17 November 2000; received in revised form 10 February 2001; accepted 5 March 2001

Abstract

We explored the evolutionary importance of two factors in the adaptation of RNA viruses to their cellular hosts, size of viral inoculum used to initiate a new infection, and mode of transmission (horizontal versus vertical). Transmission bottlenecks should occur in natural populations of viruses and their profound effects on viral adaptation have been previously documented. However, the role of transmission mode has not received the same attention. Here we used a factorial experimental design to test the combined effects of inoculum (bottleneck) size and mode of transmission in evolution of vesicular stomatitis virus (VSV) in tissue culture, and compared our results to the predictions of a recent theoretical model. Our data were in accord with basic genetic principles concerning the balance between mutation, selection and genetic drift. In particular, attenuation of vertically transmitted viruses was a consequence of the random accumulation of deleterious mutations, whereas horizontally transmitted viruses experiencing similar bottlenecks did not suffer the same fitness losses because effective bottleneck size was actually determined by the number of host individuals. In addition, high levels of viral fitness in horizontally transmitted populations were explained by competition among viral variants. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Beneficial mutations; Deleterious mutations; Experimental evolution; Fitness; Horizontal transmission; Vertical transmission; Vesicular stomatitis virus

1. Introduction

Evolution of parasite fitness is governed by competition at two different levels, intrahost replication and interhost transmission (Levin and Svanborg Éden, 1990; Bull, 1994; Ewald, 1994; Chao et al., 2000). Despite extensive theoretical work exploring the role of transmission mode in pathogen evolution (Anderson and May, 1986; Lenski and May, 1994; Lipsitch et al., 1996; Frank, 1996; Boots and Sasaki, 1999), relatively little empirical support exists for these models or their underlying assumptions (Bull et al., 1991; Herre, 1993; Turner et al., 1998; Messenger et al., 1999). Vertical transmission of parasites occurs within a single host lineage (between an infected parent and its offspring), but horizontal transmission allows a parasite to reach any susceptible host in the population regardless of descent. Therefore, competition between vertically transmitted parasites occurs only at the intrahost level, while that between horizontally transmitted parasites takes place at the interhost level as well.

Ewald (1987) suggests that virulence should decrease under vertical transmission because pathogen fitness is limited by host reproduction, a parasite that harms its host too much will reduce its own reproductive success (Chao et al. (2000) for a different prediction when interference competition occurs between parasites within the host). In contrast, vertical transmission of viral pathogens via plaque-to-plaque serial transfer in the laboratory is shown to reduce virulence through the accumulation of deleterious mutations (Domingo and Holland, 1997). In bottlenecked lineages harmful mutations will accumulate over time, causing the mean fitness of the population to decline; a process analogous to Muller's ratchet (Muller, 1964). Thus, parasite fitness can decrease through direct genetic consequences of the transmission process, rather than through a parasite's effects on host fitness.

Recently, Bergstrom et al. (1999) discussed several consequences of transmission bottlenecks for the evolution of rapidly evolving pathogens, such as RNA viruses. (i) When inoculum size is large (absence of severe bottlenecks), changes in genotype frequency caused by sampling effects become insignificant. Under vertical transmission, genotype frequencies in the independently evolving lineages will converge on an equilibrium distribution of viral phenotypes

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PII: S1567-1348(01)00006-5

present in the individual transmitting the infection. Thus, low genetic variance and similar levels of mean fitness are expected among the viral populations. By a similar argument, horizontally transmitted viruses that share a common gene pool will converge on an equilibrium phenotypic distribution (and hence the same genetic variability and fitness). But horizontal transfer allows fitter strains to export their greater productivity to other host lineages, and selection should maintain high fitness in horizontally transmitted viruses. This effect of transmission mode on fitness is expected even when the host population contains as few as five individuals. (ii) When inoculum size is small (severe bottlenecks), sampling error causes the genotype frequency distribution in a newly infected host to deviate from that in the transmitting host and, presumably, mean fitness should decline.

The evolutionary dynamics of horizontally and vertically transmitted viruses should differ in two ways. First, under vertical transmission alone the viruses in each individual host compose an evolutionarily separate population from those in every other host. Therefore, vertically transmitted viruses from different hosts at time $t = 0$ will never share a common host at time $t > 0$. In contrast, horizontally transmitted viruses from separate hosts at $t = 0$ may share a common host at $t > 0$. The result is that vertically transmitted viruses are separated into n subpopulations passing through a bottleneck of size b at each transmission event, whereas horizontally transmitted pathogens form a single population passing through a bottleneck of size nb. Second, horizontally transmitted viral populations feature a greater potential for growth rate differences to counteract mutational load. Under horizontal transmission a novel mutation will continue competing with its progenitor to infect the same set of hosts until one is lost to extinction. But with vertical transmission the two mutations can compete only as long as they reside in the same individual, and selection has less time to combat deleterious mutations. Thus, smaller bottleneck size and relatively weak selection allow mutations to more severely reduce fitness in vertically transmitted viruses than in horizontally transmitted ones.

Vesicular stomatitis virus (VSV) is a typical member of the Vesiculovirus genus (family *Rhabdoviridae*, order *Mononegavirales*), and provides an excellent model to test predictions similar to those above. VSV and other RNA viruses feature extremely high rates of spontaneous mutation (orders of magnitude larger than DNA genomes) that distinguish them from other parasites (Drake and Holland, 1999). This high mutability creates a myriad of genetic variants in the evolving population, contributing to rapid evolution in RNA viruses (Elena et al., 2000). VSV has been widely used as a model for the evolutionary genetics of RNA viruses, including studies of the fitness effects of genetic bottlenecks and Muller's ratchet (Duarte et al., 1992). In the wild, VSV readily spreads via horizontal transmission, but its vertical (transovarial) transmission has been described in arthropod vectors as well (Tesh et al., 1972; Comer et al., 1990). Therefore, both modes of transmission are a natural component of VSV biology.

Here we used VSV and eukaryotic cells in tissue culture to answer three questions. Under severe bottlenecks, can horizontally transmitted viruses better combat the mutational load and achieve greater fitness? With increased inoculum size, does the fitness of vertically transmitted viruses approach that of their horizontally transmitted counterparts? Do smaller bottlenecks lead to greater genetic variance in fitness among replicate populations regardless of transmission mode?

2. Material and methods

2.1. Viral clones, cells and culture conditions

Viruses used in this study were originally derived from the Mudd-Summer strain of the VSV Indiana serotype, hereafter referred to as wild-type (wt). MARM C is a mutant derived from wt. Both genotypes only differ on an $Asp_{259} \rightarrow Ala$ substitution in the G surface protein, a mutation that allows growth under I_1-monoclonal antibody (I_1-mAb) concentrations that completely neutralize wt (Lefrancois and Lyles, 1982; Vandepol et al., 1986). This amino acid substitution do not have any effect on MARM C fitness and both clones can be considered as effectively neutrals (Holland et al., 1991; Miralles et al., 1999, 2000). MARM C served as the common competitor in our fitness assays (see Section 2.2).

Baby hamster kidney (BHK) cells were grown as monolayers under Dulbecco modified Eagle's minimum essential medium (DMEM) supplemented with 5% newborn calf serum and 0.06% proteose peptone 3. Cells were grown to a density of $\sim 3 \times 10^5$ cells/cm^2 in 25 cm^2 plastic flasks for infections, or in 100 cm^2 plates for routine maintenance. To produce I_1-mAb, hybridoma cells were grown in DMEM containing 20% foetal bovine serum, 2 μg/ml thymidine, 0.1 mg/ml glycine, 14 μg/ml hypoxanthine and 1.3 μl/ml of Opti-mAb (GibcoBRL). All cell lines were maintained in incubators at 37°C, 5% CO_2 atmosphere, and 95% relative humidity.

2.2. Experimental design

Our experimental design (Fig. 1) mimicked virus evolution in a population of multicellular hosts reproducing through non-overlapping generations. Each "host individual" was a 25 cm^2 flask containing $\sim 7.5 \times 10^6$ cells, and host populations contained five individuals. On day one of the experiment we infected 20 flasks with $\sim 2 \times 10^5$ plaque forming units (PFU) of wt. After 24 h incubation all cells lysed, producing $\sim 5 \times 10^{10}$ PFU. The 20 flasks were then divided into four treatments, horizontal transmission via large bottleneck (HL), horizontal transmission via small bottleneck (HS), vertical transmission via large bottleneck (VL), and vertical transmission via small bottleneck (VS).

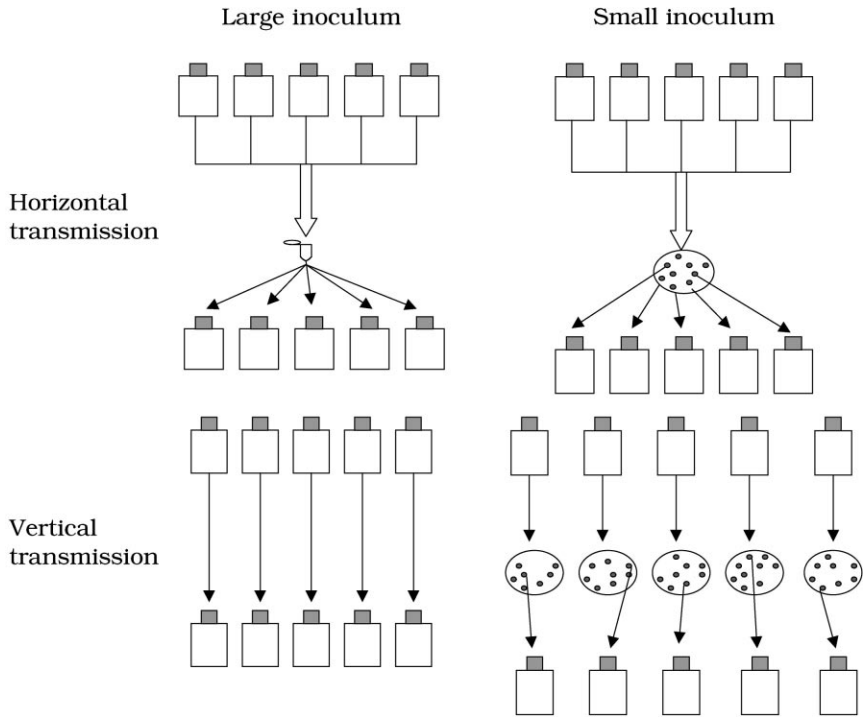

Fig. 1. Summary of the experimental protocol for each treatment. Populations experienced either horizontal or vertical transmission with small or large inoculum size. Each group contained five flasks, and the entire experiment was replicated four-fold, (see Section 2).

In the HL treatment, viral progeny from all five flasks were pooled, diluted 10^4-fold, and $\sim 2 \times 10^5$ PFU were used to infect each of five new host flasks. This ensured that viral progeny from independent lineages were transmitted horizontally to a new susceptible host regardless of descent. In the HS treatment, viral progeny were pooled as above, but the diluted mixture was plated on a cell monolayer containing agarose in the overlay medium. After 24 h incubation, this produced visible plaques (each containing $\sim 5 \times 10^3$ PFU) initiated through infection of only a single virus. We chose five plaques at random, diluted each in 1 ml of DMEM and used this to infect a new host flask. Thus, horizontal transmission occurred as above, but bottleneck size in the HS treatment was only one PFU per flask. In the VL treatment, viral progeny from each flask were diluted 10^4-fold, and $\sim 2 \times 10^5$ PFU were used to infect a new host flask. This allowed the VL lineages to evolve strictly through vertical transmission. In the VS treatment, viral progeny from each host flask were diluted separately and plated as above. One plaque was chosen at random from each plate, diluted in 1 ml of DMEM, and used to infect a new host flask. This ensured strictly vertical transmission, but a bottleneck size of only one PFU.

Are the differences between our large and small populations biologically meaningful? Single virion bottlenecks are probably very common during respiratory transmission of viruses because most respiratory droplets should contain very few or no infectious particles (Couch et al., 1966). Transmission of HIV-1 by infectious virions might often involve only one or several infectious particles (Pang et al., 1992) whereas transmission by infected cells (e.g. hemophiliacs transfused with contaminated blood or drug abusers sharing syringes) might involve numerous infectious genomes (Lukashov and Goudsmit, 1997). In addition, routines for keeping viral populations in laboratory conditions, including techniques for producing vaccines by virus attenuation in naïve hosts, usually involved from one (plaque cloning) to several thousand particles (massive transfers) per transmission event (Enders et al., 1962; Sabin and Boulger, 1973).

Because all experimental infections involved naïve host cells, this prevented evolution of host resistance and coevolution between viruses and hosts. The HL and VL treatments allowed four generations of viral evolution per day (Miralles et al., 2000), whereas eight generations occurred in the 2-day cycle of the HS and VS treatments. To achieve 80 generations of evolution across all treatments, we conducted 20 consecutive transfers in the HL and VL treatments and 10 transfers in the HS and VS treatments.

For greater statistical power, the entire experiment was replicated four-fold in independent blocks. Because the number of viruses per cell never exceeded 0.03 in our treatment populations, this eliminated any confounding effects due to evolution of defective-interfering particles (Horodyski et al., 1983), or intracellular interactions among competing genotypes (Turner and Chao, 1998).

2.3. Relative fitness assays

To measure relative fitness (Holland et al., 1991), each evolved wt population was mixed with a known amount of MARM C, and the initial ratio (R_0) was determined by plating with and without I_1-mAb in the agarose overlay medium. (Incorporation of the antibody into the plaque overlay medium (after virus penetration), instead of standard virus neutralization, avoids the encapsidation of MARM RNAs within phenotypically wt envelopes, a problem known as phenotypic mixing and hiding (Holland et al., 1989)). The resulting virus mixture was diluted 10^4-fold and used to initiate the next competition passage by infection of a fresh monolayer. We then serially transferred competition mixtures for up to three passages to obtain good estimates of relative fitness. Samples from competition mixtures determined the ratio of wt to MARM C at transfer t (R_t). The antilogarithm of the slope of the regression $\ln R_t = \ln R_0 + t \ln W$ was used to estimate fitness (W) of the wt competitor relative to MARM C (Elena et al., 1998).

2.4. Statistical analysis

The linear model used to fit the data was a mixed model II ANOVA where the random factor "individual host" (flask) was nested within each treatment (HL, HS, VL and VS). The entire experiment was replicated in four independent blocks, allowing the two fixed factors (transmission type and inoculum size) and their interaction to be nested within block. Here, a fitness value (W) is defined as the mth fitness determination for individual host l ($l = 1, \ldots, 5$), at inoculum size j (j = L, S) and transmission mode i (i = H, V) in experimental block k ($k = 1, \ldots, 4$). This yields the expression

$$W_{ijklm} = \mu + B_k + T_{ki} + I_{kj} + (TI)_{kij} + F_{kijl} + \xi_{kijlm}$$

where μ is overall mean fitness, B block effect, T effect of transmission mode within a block, I effect of inoculum size within a block, TI the interaction between transmission mode and inoculum size within a block, and F effect of an individual host within block. Finally, ξ_{kijlm} an error term. It is assumed that B, T, I, F and ξ are normally distributed with mean zero and positive variance (Sokal and Rohlf, 1995).

Some analyses tested the effect of only one fixed factor (transmission mode or inoculum size), simplifying the above expression to $W_{ijkl} = \mu + B_k + X_{ki} + F_{kij} + \xi_{kijl}$, where X the factor considered.

To test predictions regarding the degree of genetic differentiation between replicate viral populations, we estimated among-flasks within-block variance for fitness, σ_F^2, using a maximum likelihood method (Lynch and Walsh, 1998). To facilitate statistical comparisons between estimates of σ_F^2, 95% confidence intervals were calculated. Broad sense heritability, H^2, was employed to measure the extent of genetic divergence relative to the total observed variance.

All statistical analyses described were carried out using SPSS 10 for Windows (SPSS Inc., 2000).

3. Results

3.1. Preliminary analyses

Preliminary fitness assays ($n = 20$) determined the mean fitness of wt relative to MARM C to be 0.9848 ± 0.1203 SEM, a value not statistically different from 1.0 ($t_{19} = 0.1264$, $P = 0.9008$). This result confirmed that the MARM C common competitor is neutral relative to wt (Holland et al., 1991).

Prior to data analysis, we tested the assumptions of ANOVA. Two key assumptions were not met, the data were not normally distributed (Kolmogorov–Smirnov's $D = 0.1755$, $Z = 2.7686$, $P < 0.0001$) and heteroscedasticity among groups was detected (Levene's $F_{79,169} = 3.1272$, $P < 0.0001$). The data were normalized by transformation using a Box–Cox power function (maximum likelihood estimate for the power parameter $\hat{\lambda} = 0.1605$, with 95% confidence interval $0.0357 \leq \hat{\lambda} \leq 0.2548$ (Sokal and Rohlf, 1995)). All statistical tests reported below were computed with the transformed data. However, to preserve biological meaning, mean fitness values are reported without transformation.

Table 1 shows the results of the ANOVA. Three major conclusions can be drawn regarding the fixed effects in our experiment. First, effect of transmission mode on fitness was found to be not significant. Second, effect of inoculum size on fitness was found to be significant, indicating that viruses evolved via large inocula systematically attained higher fitness (see Section 3.3). Third, and most important, we observed a highly significant interaction between transmission mode and inoculum size. This result was due to large differences in fitness between horizontally and vertically transmitted populations at small inoculum sizes (see Section 3.2).

Table 1
Mixed model II analysis of variance[a]

Source of variation	SS[b]	d.f.	MS	F	P
Block	33.5304	3	11.1768	0.3463	0.7946
Transmission mode	23.7861	4	5.9465	1.5355	0.3440
Inoculum size	120.8866	4	30.2216	7.8035	0.0358
Transmission mode × inoculum size	15.4912	4	3.8728	6.8804	0.0001
Individual host	36.1675	64	0.5651	1.7968	0.0015
Error	53.1541	169	0.3145		

[a] Mode of transmission and inoculum size were treated as fixed factors, whereas replicate experiments (blocks) and individual hosts (flasks) were treated as random factors (see text for description of the linear model fitted). Fitness values were transformed using a Box–Cox power function to normalize these data and reduce heteroscedasticity of variances (see Section 3) more appropriate.

[b] SS is the type III error sum of squares.

Regarding random factors, we observed homogeneity among the four replicate blocks of our experiment and, more interestingly, significant heterogeneity among the five individual hosts (flasks) within each block. These differences among individual flasks may not be surprising given the high mutation rates characteristic of RNA viruses in general and VSV in particular (Drake and Holland, 1999; Elena et al., 2000). Furthermore, as described in the Section 1, two additional factors can play a role in the origin and maintenance of genetic differences among viral lineages: vertical transmission, and strong random drift that is associated with bottlenecks of size one at small inocula. We consider these phenomena more fully in the next sections.

3.2. Effect of transmission mode under severe bottlenecks

We hypothesized that in the presence of severe bottlenecks horizontally transmitted viruses are better able to combat mutational load and hence, achieve higher fitness levels than vertically transmitted viruses. Of issue is (i) evidence for mutational load in our experiments, and (ii) demonstration that horizontal transmission combats the load.

We first sought to establish whether our viral populations accumulate a mutational load (experience reduced fitness) when bottlenecks are severe. To that end, we measured fitness relative to MARM C for each of the 20 VS populations with replication ($n = 3$). The grand mean of these data (Fig. 2), 0.7490 ± 0.0978, was found to be significantly <1.0 ($t_{19} = 2.5677$, 1-tail $P = 0.0094$), demonstrating the expected negative effect on viral fitness of continued bottlenecks.

We hypothesized that horizontal transmission would reduce the negative effect of small inocula on viral fitness. To test this idea we measured the fitness of each HS population relative to MARM C with replication ($n = 3$). The resultant grand mean (Fig. 2), 2.0714 ± 0.2224, was shown to be significantly greater than 1.0 ($t_{19} = 4.8173$, 1-tail $P < 0.0001$). More importantly, data for the HS populations were found to be significantly greater than those obtained for the VS populations (nested ANOVA: $F_{4,32} = 17.3615$, 1-tail $P < 0.0001$). Thus, not only did horizontal transmission allow HS viruses to overcome the mutational load, but it also promoted fitness improvements in these populations.

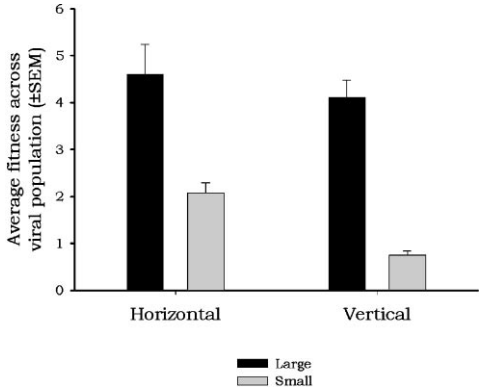

Fig. 2. Effect of transmission mode (vertical vs. horizontal) and inoculum size (small vs. large) on the extent of VSV adaptation to the cellular host. Each bar is the grand mean of four blocks containing five replicate populations. Error bars represent standard errors of the mean.

3.3. Genetic variation under severe bottlenecks

In the presence of severe bottlenecks, sampling error becomes important. We hypothesized that genetic variance in VS populations should be greater than that in VL populations. Results in Table 2 supported this idea, σ_F^2 estimated for the VS group was significantly greater than that for the VL group (where the latter variance was immeasurably small), and represented a higher percentage of the observed variability (i.e. greater H^2). This contrasts with our observation that fitness of VL populations significantly exceeded that of VS ones (nested ANOVA: $F_{4,32} = 48.5728$, $P < 0.0001$).

In contrast, bottlenecks are expected to have less of an effect on genetic variance in horizontally transmitted populations. Therefore, σ_F^2 in HL and HS groups was expected to be similar in magnitude. But our results were not supportive, σ_F^2 in HL populations significantly exceeded that in HS populations (Table 2) and also represented a larger proportion of the total observed variability. Similarly, fitness of HL populations was higher than that of the HS group (see Section 3.2), and the difference was significant (nested ANOVA: $F_{4,32} = 11.3177$, $P < 0.0001$).

It is also likely that severe bottlenecks should have a stronger effect on genetic variability of vertically transmitted viruses than horizontally transmitted ones, greater variation is expected among vertically transmitted viruses due to the isolation of lineages. This idea was supported by the results shown in Table 2, σ_F^2 in the VS group was significantly greater than that in the HS group, and accounted for more of the observed variability.

Table 2
Variance among replicate viral populations, σ_F^2, estimated for each experimental group[a]

Transmission mode	Inoculum size	
	Small	Large
Horizontal	$\hat{\sigma}_F^2 = 0.0370$ $0.0231 \le \hat{\sigma}_F^2 \le 0.0509$ $H^2 = 8.09\%$	$\hat{\sigma}_F^2 = 0.1383$ $0.0231 \le \hat{\sigma}_F^2 \le 0.0509$ $H^2 = 21.02\%$
Vertical	$\hat{\sigma}_F^2 = 0.1530$ $0.1264 \le \hat{\sigma}_F^2 \le 0.1795$ $H^2 = 33.97\%$	$\hat{\sigma}_F^2 = 0$ $H^2 = 0$

[a] 95% confidence intervals were computed from the error of the maximum likelihood estimates of $\hat{\sigma}_F^2$. Broad sense heritability for fitness, H^2, is also provided.

3.4. Effect of transmission mode in the absence of bottlenecks

We next tested whether absence of bottlenecks allows the fitness of vertically and horizontally transmitted viruses to converge. To do so, we measured fitness relative to MARM C for each population in the VL and HL treatments with replication ($n = 3$, Fig. 2). The grand mean fitness for the 20 HL populations was 4.5975 ± 0.6423, a value significantly greater than 1.0 ($t_{19} = 5.6009$, 1-tail $P < 0.0001$). Similarly, the 20 VL populations showed a grand mean fitness of 4.1060 ± 0.3752, a value that also exceeded 1.0 ($t_{19} = 8.2782$, 1-tail $P < 0.0001$). We observed no significant effect of transmission mode on fitness in these data (nested ANOVA: $F_{4,32} = 0.6182$, $P = 0.6527$), strongly suggesting that the reduction in viral fitness observed in the VS populations was solely due to differences in inoculum size. In contrast, large inoculum sizes allowed fitness of vertically transmitted viruses to approach that of their horizontally transmitted counterparts.

3.5. Genetic variation in the absence of bottlenecks

Absence of bottlenecks causes sampling effects to become insignificant, regardless of transmission mode. Therefore, we expected low genetic variance and similar fitness gains within the VL group, and within the HL group. Results (Table 2) showed σ_F^2 in the VL group to be null (immeasurably small). Not surprisingly, a one-way ANOVA showed that VL populations did not differ significantly in mean fitness ($F_{16,42} = 0.9942$, $P = 0.4807$). Genetic variance for populations in the HL group represented $H^2 \approx 21\%$ of the total observed variability (Table 2), but the 95% confidence interval for variance excluded zero, indicating that σ_F^2 for the HL group is significantly greater than that of the VL group. Consequently, the HL populations differed in mean fitness (ANOVA with $F_{16,42} = 2.3192$, $P = 0.0145$).

As shown above, the grand mean fitness of VL populations approached that of HL populations, and an ANOVA showed no compelling differences between modes of transmission when population size was large. However, confidence intervals for σ_F^2 did not overlap for these two groups (Table 2), indicating significantly greater genetic variance in the HL group. We concluded that absence of bottlenecks allowed populations in the VL group to approach fitness values attained by the HL viruses, but that the two groups differed in extent of genetic variability.

4. Discussion

Evidence for viral attenuation in the face of severe bottlenecks has been previously reported for VSV (Duarte et al., 1992) and other viruses (Chao, 1990; Escarmís et al., 1996; Yuste et al., 1999; de la Peña et al., 2000). Following this precedent, our experiment yields new insights into the importance of transmission mode in combating mutational meltdown. In general agreement with the predictions of a recent model by Bergstrom et al. (1999), our results showed that severe bottlenecks led to attenuation in vertically transmitted viruses, but not in their horizontally transmitted counterparts. In addition, no effect of transmission mode was observed in the absence of bottlenecks. These data matched the model's predictions regarding fitness changes in rapidly evolving populations. We also tested the model's predictions regarding genetic variation among viral lineages, and our empirical data fit two of these predictions. (i) Vertical transmission increased genetic variance when drift was allowed to act (VS), in contrast, when the effects of drift were minimized by large inoculum sizes, decreased genetic variance was observed (VL). (ii) Small inoculum size generated greater variance in vertically (VS) than in horizontally transmitted viruses (HS). However, our results did not match two other predictions of the model. (i) In horizontally transmitted viruses we found that greater genetic variance occurred when population size was large (HL) relative to small (HS). (ii) We found dissimilarities in genetic variance between horizontally and vertically transmitted viruses at large population sizes (i.e. we observed greater genetic variance for HL populations relative to VL ones).

In RNA virus populations, mutations occur at a very high rate, roughly 1–3 per genome and generation (Drake and Holland, 1999). Of mutations generated, the majority are deleterious (Elena and Moya, 1999), and only a tiny fraction are beneficial (Miralles et al., 1999). If population size is large, the major factor that drives viral evolution is selection acting on rare beneficial mutations, and the outcome of competition between genomes carrying different beneficial mutations is the fixation of the best possible candidate (Miralles et al., 1999) regardless of transmission mode. In contrast, when population size is small, random genetic drift will be the major evolutionary force and any remaining soft selection is not efficient in eliminating deleterious mutations. Under the latter circumstance, the dynamics of viral transmission will play an important role. If transmission occurs horizontally, different genotypes compete within hosts and the most fit is expected to spread to fixation. In contrast, strictly vertical transmission prevents competition between different lineages and deleterious mutations can easily accumulate, with consequent reduction in fitness (Chao, 1990; Duarte et al., 1992; Escarmís et al., 1996; Yuste et al., 1999; de la Peña et al., 2000). With tighter bottlenecks, natural selection should be less efficient and fitness should decline, as previously observed for VSV (Novella et al., 1995, 1996).

Our results provide empirical evidence for attenuation of vertically transmitted viruses in the absence of feedbacks on host fitness. In lytic viruses, virulence and fitness are generally tightly coupled. Attenuation of vertically transmitted RNA viruses can result from two different genetic forces acting on virus populations, and not solely due to genetic changes or physiological responses in the host as previously

postulated (Ewald, 1987). First, when bottlenecks are severe and mutation rate is high, Muller's ratchet pushes viral fitness downward due to loss of the least mutated genotypic class. Second, in vertically transmitted viruses different viral genotypes are separated into distinct evolutionary lineages, reducing the level of competition and, hence, the ability for natural selection to improve viral fitness.

A variety of models, within the framework of evolutionary ecology of infectious diseases, seek to explain evolution of pathogen fitness in light of transmission dynamics (Knolle, 1989; Nowak, 1991; Yamamura, 1993; Lipsitch and Nowak, 1995; Lipsitch et al., 1996; Boots and Sasaki, 1999). But, despite their explanatory power, these models are weak in one respect, lack of a genetic basis for model parameters. For instance, what is the genetic basis for a trade-off between parasite virulence and transmission rate? What is the molecular basis for attenuation of pathogen virulence? In contrast, the model proposed by Bergstrom et al. (1999) incorporates well-characterized genetic parameters: mutation, selection and drift. Furthermore, their model's predictions are easily testable, as we have demonstrated.

Although, we empirically tested several of the predictions made by Bergstrom et al. (1999), others remain unexplored. For example, the role of host population size in the evolution of viral fitness. The model predicts that host population size should have no effect when transmission is primarily vertical, because viruses within each lineage are unaffected by the total number of lineages. However, if transmission occurs horizontally, the effective bottleneck size increases with the number of available hosts, suggesting that viral fitness should positively correlate with host population size (also predicted by Boots and Sasaki, 1999) Ecological models, however, predict that when parasites reduce the number of susceptible hosts, intermediate levels of virulence can be achieved even by vertically transmitted parasites (Lenski and May, 1994; Lipsitch and Nowak, 1995). These opposing predictions can be empirically tested through viral evolution experiments similar to the ones reported here, and this provides a motivation for future research.

The results here presented suggest that the balance between vertical and horizontal transmission during an epidemic will strongly influence the fitness and virulence of a viral population. For example, let us consider the case of HIV-1. During the early moments of the pandemic, horizontal transmission, mainly by a promiscuous sexual behavior, was the main cause for HIV-1 spread (Ewald, 1992). It has been suggested that the virulence of HIV-1 should be correlated with the rate of sexual contact (Ewald, 1992; Massad, 1996) and, consequently, it was high at the beginning. However, as prophylactic measures are being taken worldwide, extending the use of condoms to avoid person-to-person (horizontal) transmission, the relative importance of vertical mother-to-child transmission will raise up (European Collaborative Study, 1992). Therefore, if the predictions of several models hold (Bergstrom et al., 1999; Lipsitch and Nowak, 1995; Lipsitch et al., 1996; Yamamura, 1993), it is expected that, as a consequence of a new predominant way of transmission, we will assist to a future attenuation of HIV-1 virulence. Only time will confirm or refuse this expectation.

Acknowledgements

We thank C. Bergstrom and R. Miralles for helpful comments or suggestions, and O. Cuesta for excellent technical assistance. This research was supported by grant PM97-0060-C02-02 from Spanish Ministerio de Educación y Cultura and by grant 1FD1997-2328 from the European Union. P.E.T. was supported by a National Science Foundation-North Atlantic Treaty Organization postdoctoral fellowship. R.S. received a research training fellowship for undergrad students from the Spanish Ministerio de Educación y Cultura.

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