JOURNAL OF VIROLOGY, May 1993, p. 2938–2943
0022-538X/93/052938-06$02.00/0
Copyright © 1993, American Society for Microbiology
Vol. 67, No. 5

Dilute Passage Promotes Expression of Genetic and Phenotypic Variants of Human Immunodeficiency Virus Type 1 in Cell Culture

S. SÁNCHEZ-PALOMINO,[1] J. M. ROJAS,[1] M. A. MARTÍNEZ,[2] E. M. FENYÖ,[3] R. NÁJERA,[1] E. DOMINGO,[2] AND C. LÓPEZ-GALÍNDEZ[1]*

Centro de Biología Celular y Retrovirus, Instituto de Salud Carlos III, Majadahonda, 28220 Madrid,[1] and Centro de Biología Molecular "Severo Ochoa," CSIC-UAM, Universidad Autónoma, Cantoblanco, 28049 Madrid,[2] Spain, and Department of Virology, Karolinska Institute, Stockholm, Sweden[3]

Received 1 October 1992/Accepted 22 January 1993

We have studied the extent of genetic and phenotypic diversification of human immunodeficiency virus type 1 (HIV-1) upon 15 serial passages of clonal viral populations in MT-4 cell cultures. Several genetic and phenotypic modifications previously noted during evolution of HIV-1 in infected humans were also observed upon passages of the virus in cell culture. Notably, the transition from non-syncytium-inducing to syncytium-inducing phenotype (previously observed during disease progression) and fixation of amino acid substitutions at the main antigenic loop V3 of gp120 were observed in the course of replication of the virus in MT-4 cell cultures in the absence of immune selection. Interestingly, most genetic and phenotypic alterations occurred upon passage of the virus at a low multiplicity of infection (0.001 infectious particles per cell) rather than at a higher multiplicity of infection (0.1 infectious particles per cell). The degree of genetic diversification attained by HIV-1, estimated by the RNase A mismatch cleavage method and by nucleotide sequencing, is of about 0.03% of genomic sites mutated after 15 serial passages. This value is not significantly different from previous estimates for foot-and-mouth disease virus when subjected to a similar process and analysis. We conclude that several genetic and phenotypic modifications of HIV-1 previously observed in vivo occur also in the constant environment provided by a cell culture system. Dilute passage promotes in a highly significant way the expression of deviant HIV-1 genomes.

An extensive genetic and antigenic variability has been documented for retroviruses generally (7, 8) and particularly for human immunodeficiency virus type 1 (HIV-1) (3, 6, 7, 23, 30, 32, 38, 39, 42, 48), the causative agent of AIDS. HIV-1 populations, like those of other RNA viruses, are composed of swarms of related genomes termed viral quasispecies (14, 17, 25, 26, 32, 45). Genetic heterogeneity is a consequence of the high mutation rates operating during replication of HIV-1 which dictate that in any HIV-1 population, many different phenotypic variants with altered replicative and cytopathic properties may coexist (19, 22). Shifts between viral subpopulations sequentially found in infected individuals (32) may be triggered by selective forces acting on certain genomes; alternatively, they may result from the random sampling of genomes that become dominant irrespective of any biological features that they might display. Wain-Hobson and colleagues have suggested that HIV-1 variants initially present as a minority in the population may be amplified from proviruses by any antigenic stimulation of lymphocytes and other lymphoid cells, leading to modifications of HIV-1 quasispecies independent of the actual competitive fitness of the variants involved (9, 45). Other factors that modulate the evolution of viral populations are sampling events (47) and repeated bottleneck transmissions (5, 16).

Differences in replicative capacities of HIV-1 isolates have been described (for a review, see reference 19). Viruses able to replicate in T-lymphoid and monocytoid cell lines and able to form syncytia in peripheral blood mononuclear cell (PBMC) cultures are isolated from patients with severe immunodeficiency. In contrast, viruses isolated from individuals with no or mild symptoms of HIV-1 infection usually lack these properties. Increase in replicative capacity and emergence of syncytium-inducing (SI) virus appears to be linked to HIV-1 pathogenesis, inasmuch as the transition from dominance of slowly replicating, non-syncytium-inducing (NSI) viruses to dominance of high-replicating, SI variants correlates with disease progression (1, 6, 43). It has been suggested that selection of variants that are more pathogenic and show altered cell tropism may be concomitant with the gradual decrease in the number of $CD4^+$ T cells and the weakening of the immune response in infected individuals (43).

The objective of this study was to analyze and quantitate the genetic and phenotypic diversification of carefully isolated clones of HIV-1 in cell culture, without many of the perturbations unavoidable in infected organisms. Following previous designs of experiments on clonal heterogeneity of serially passaged phage (15) and animal virus populations (40), we have analyzed the extent of genetic and phenotypic diversification of clonal populations of HIV-1 serially passaged in MT-4 cell cultures but at two different multiplicities of infection (MOIs). Interestingly, several of the variant phenotypes previously described in isolates from AIDS patients, as well as transitions between distinct phenotypes, were readily observed upon passage of HIV-1 under the controlled environment provided by this cell culture system.

The viral clones analyzed were derived from HIV-1-61, an isolate obtained in 1989 from a 4-year-old boy in Madrid, Spain, who manifested symptoms at stage P2CD2 (Centers for Disease Control classification). HIV-1-61 was isolated by

* Corresponding author.

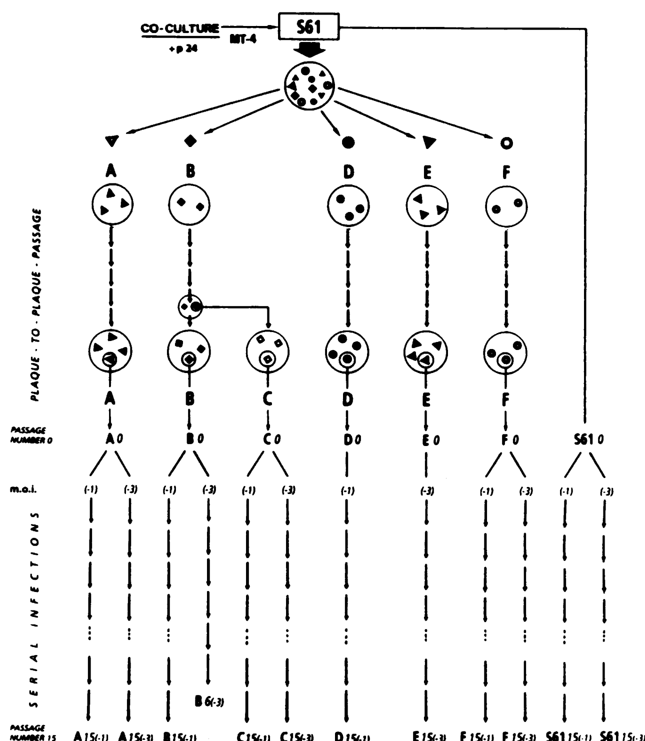

FIG. 1. Scheme of five consecutive plaque-to-plaque isolations and 15 serial passages of clonal populations of HIV-1. The supernatant from the coculture of HIV-1-61 with PBMC was used to directly infect MT-4 cells. The resulting virus preparation, S61, was either serially passaged in MT-4 cells or diluted and plated for the isolation of individual plaques as described previously (24), with some modifications. Briefly, an appropriate dilution of the medium containing progeny virus was plated on MT-4 cell monolayers; after a 1-h adsorption period at 37°C, the inoculum was removed, and an agarose (SeaPlaque; Marine Colloid Corp.) overlay was added. Following a 3-day incubation at 37°C in 5% CO_2, a second agarose overlay was added, and upon an additional incubation period of 6 days, plaques were visible without staining. Virus was picked from five well-isolated plaques (A, B, D, E, and F) chosen at random, diluted, and further subjected to a total of five successive plaque isolations. Clone C was derived from the penultimate plaque isolation from series B. Plaque assays to determine PFU titers for the MOIs were carried out in triplicate. After the last cloning step, the plaqued viruses were amplified by infection of MT-4 cells to produce preparations A0, B0, C0, D0, E0, and F0. Then each viral clone and S61 were serially passaged 15 times by infecting MT-4 cells in suspension at an MOI of 0.001 PFU per cell (in each passage, 5×10^3 PFU was used to infect 5×10^6 cells) and/or 0.1 PFU per cell (5×10^5 PFU to 5×10^6 cells). Viral populations are designated with the letter corresponding to the initial clone, followed by passage number and the logarithm of the MOI used; i.e., F15(−3) is the population resulting from 15 serial passages of clone F0 at an MOI of 0.001 PFU per cell. The initial and final virus populations were subjected to several analyses described in the text.

standard coculture procedures. The medium of such culture (positive for p24 antigen) was used directly to infect MT-4 cells to yield S61, the initial viral population of this study (Fig. 1). Then biological clones were obtained by agarose plaque purification (24) from the global population and differentiated by RNase A digestion pattern (29, 30). One clone representative of each genomic pattern (populations A0, B0, D0, E0, and F0) and in addition C0, which was derived as a subline of B (Fig. 1), were chosen for further studies. Viruses were titrated in the MT-4 plaque assay, and passages in cell cultures were performed at an MOI of 0.1 or 0.001 PFU per cell. Infections were monitored by cytopathic effect (CPE), determination of cell viability, or indirect immunofluorescence using a pool of human sera positive for HIV-1 and, when necessary, by p24 antigen determination. Infections were also monitored by measuring the reverse transcriptase (RT) activity of virus in the culture medium as previously described (46).

The viral clones obtained by plaque purification and the populations resulting from 15 serial passages of clones on MT-4 cells were characterized for replication in primary cells (PBMC and monocyte/macrophages) as well as in cell lines (HuT-78 and U937 clone 2) (20). PBMC from normal blood donors were separated on Ficoll-Paque (Pharmacia) and stimulated with 2.5 mg of phytohemagglutinin (PHA-P; Difco) per ml for 3 days prior to infection. Monocyte/macrophage cultures were prepared as previously described (44). In short, 2.5×10^7 PBMC were seeded into 25-cm^2 plastic culture flasks in RPMI medium supplemented with 10% heat-inactivated pooled HIV-1-negative human serum and 20% fetal calf serum (FCS) and incubated for 5 days at 37°C. Cultures were then washed extensively with phosphate-buffered saline to remove nonadherent cells and maintained in RPMI medium containing 20% FCS. Cell lines were grown in RPMI 1640 medium (GIBCO) supplemented with 10% FCS, 2 mg of Polybrene per ml, and antibiotics. In addition, PBMC cultures were supplemented with 5 U of recombinant interleukin-2 (Amersham) per ml. Supernatants of infected cultures were tested for virus production by the RT assay and p24 antigen enzyme-linked immunosorbent assay (ELISA) (41) twice a week for 4 weeks.

Total RNA was extracted from the infected cells and analyzed by the RNase A mismatch cleavage method (29, 30) (Fig. 2). The RNase A mismatch cleavage method was applied to the following genomic segments: GAG (positions 1259 to 1645), 5′-end domain of RT (positions 2202 to 2562, designated RT5′); 3′-end domain of RT (positions 2559 to 2884, designated RT3′); VIF-VPR (4704 to 5372), and ENV (7199 to 7723) [numbering according to that of Ratner et al. (35) for HIV-1 (IIIB)]. The analysis was carried out as previously described (29, 30). For polymerase chain reaction amplification of the genomic RNA region encoding the V3 domain of gp120, primers corresponding to positions 6642 to 6665 (35) and complementary to positions 6913 to 6893 (35) were used to amplify cDNA. The products were sequenced by dideoxynucleotide chain termination with T7 DNA polymerase (27). The primers for amplification and polymerase chain reaction sequencing (37) of the proviral DNA sequence surrounding the cleavage site and the fusion domain of gp41 corresponded to positions 7229 to 7250 (35) and positions 7919 to 7941 (35).

Genetic differences among HIV-1 clones. Initially, five plaque-purified clones were subjected to five plaque-to-plaque passages (Fig. 1); then six viral clones were selected according to variance in the RNase A mismatch cleavage method (29). Five of them could be readily differentiated according to their digestion patterns; the exception, clone C, was studied despite being indistinguishable from clone B. Clones A0, B0, and C0 showed a common pattern with all of the probes except that clone A0 differed specifically in the profile given by the RT 3′ probe (Fig. 2). Clones D0 and E0 displayed two different digestion patterns in the GAG probe. Clone F0 was distinguished by additional bands with use of the VIF-VPR probe. All of these differences are highlighted by arrowheads in Fig. 2. The five genomic probes used (30)

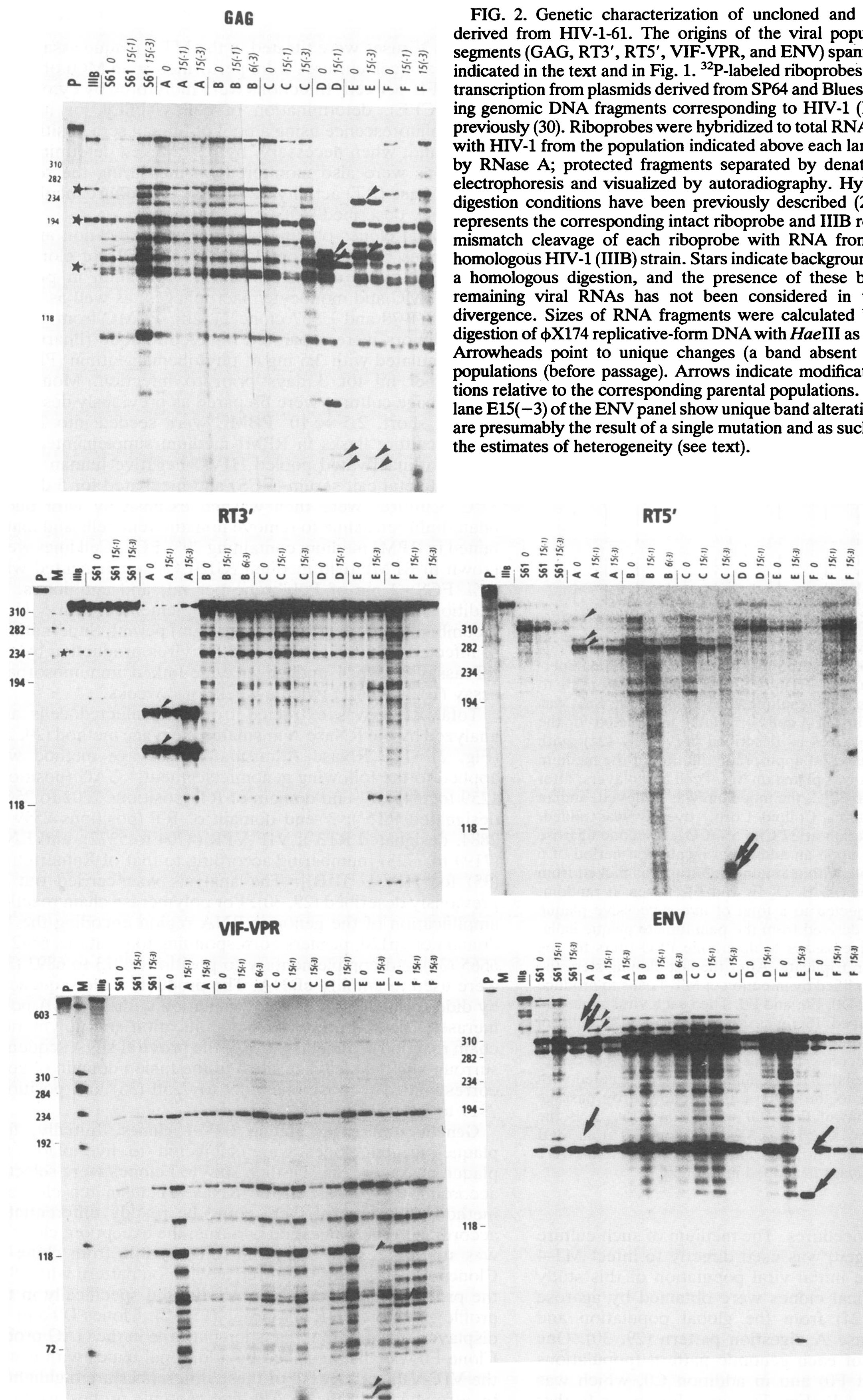

FIG. 2. Genetic characterization of uncloned and cloned viral populations derived from HIV-1-61. The origins of the viral populations and the genomic segments (GAG, RT3′, RT5′, VIF-VPR, and ENV) spanned by each riboprobe are indicated in the text and in Fig. 1. ^{32}P-labeled riboprobes were prepared by in vitro transcription from plasmids derived from SP64 and Bluescript (Stratagene) containing genomic DNA fragments corresponding to HIV-1 (IIIB) that were described previously (30). Riboprobes were hybridized to total RNA from MT-4 cells infected with HIV-1 from the population indicated above each lane. Hybrids were digested by RNase A; protected fragments separated by denaturing polyacrylamide gel electrophoresis and visualized by autoradiography. Hybridization and RNase A digestion conditions have been previously described (29, 30). In each panel, P represents the corresponding intact riboprobe and IIIB represents the result of the mismatch cleavage of each riboprobe with RNA from cells infected with the homologous HIV-1 (IIIB) strain. Stars indicate background bands produced in such a homologous digestion, and the presence of these bands in digestion of the remaining viral RNAs has not been considered in the estimates of genetic divergence. Sizes of RNA fragments were calculated by using the products of digestion of ϕX174 replicative-form DNA with *Hae*III as markers (indicated by M). Arrowheads point to unique changes (a band absent or present) in the initial populations (before passage). Arrows indicate modifications of passaged populations relative to the corresponding parental populations. The two arrows shown in lane E15(−3) of the ENV panel show unique band alterations for this sample, which are presumably the result of a single mutation and as such have been considered in the estimates of heterogeneity (see text).

TABLE 1. Summary of changes occurring in the global viral population and individual clones during in vitro cultivation

Virus	Change at an MOI of [a]:					
	0.1 PFU/cell	0.001 PFU/cell				
	(Replication)[b]	CPE[c]	Replication	Mismatch[d] RT5′	Mismatch[d] ENV	V3 sequence[e]
S61	·	·	·	·	+++	+
A	·	·	+	·	·	·
B	·	·	·	·	·	·
C	·	·	·	++	·	·
D	+	ND[f]	ND	ND	ND	ND
E	ND	·	+	·	+	+
F	·	NSI→SI	·	·	·	+

[a] +, change; · no change.
[b] Alterations observed in the RT replication assay (46).
[c] Variations in the type of CPE observed in MT-4 cells (NSI or SI).
[d] Differences in the RNase A digestion patterns using riboprobes corresponding to RT5′ and Env, the only ones in which differences were detected; each plus sign represents a band difference (see Fig. 2).
[e] Populations in which an amino acid substitution was found in the principal neutralizing epitope V3.
[f] ND, not done.

in this analysis sampled a total of 2,263 nucleotides, representing about 25% of the genome length.

Genetic variation of HIV-1 upon 15 serial passages of clonal viral populations. Band differences between RNA from viral populations passaged 15 times and from the corresponding initial clones (arrows in Fig. 2) were observed in the RT 5′ segment of clone C as well as in the *env* gene of S61 and of clone E. All of these band differences (Fig. 2) occurred in populations passaged at an MOI of 0.001 PFU per cell but not in parallel passages at an MOI of 0.1 PFU per cell. However, population E passaged at an MOI of 0.1 was not examined. For quantification of the number of mutations needed to account for the differences in the band patterns, it must be considered that under the cleavage conditions used, it has been estimated that about 60% of mismatches are recognized and cleaved by RNase A (29, 34). Assuming that the loss or gain of one band represents at least one point mutation, the passaged populations must differ from each respective parental clones on an average of at least 0.02% of nucleotides.

To further quantitate the extent of genetic diversification, we sequenced the RNA segments around the V3 domain of gp120 and the fusion domain of gp41. No mutations were found in 200 nucleotides of the DNA encoding the fusion domain of gp41 in any of the clones or passaged populations. In the V3 loop of gp120, three nucleotides changes, each giving rise to an amino acid substitution, were detected (data not shown). The total number of mutations found in these sequences amounts to 0.04% of nucleotides screened, a value which is in good agreement with the results of the RNase A mismatch cleavage method. Again, the replacements occurred in populations passaged at a low MOI of 0.001 PFU per cell, and no genetic modifications were observed at an MOI of 0.1 PFU per cell (Table 1).

Phenotypic differences and alterations upon serial passage of clonal populations of HIV-1. The populations serially passaged in cell culture were analyzed with regard to their ability to form syncytia upon infection of MT-4 cells, replication rate, and ability to infect macrophages, PBMC, and U937 clone 2 cells.

In MT-4 cells, the initial S61 population and clones A0, B0, C0, D0, and E0 induced the formation of syncytia (SI phenotype). In contrast, F0 produced CPE without syncytium formation (NSI phenotype) (data not shown). However, serial passage of population F0 (NSI) at a low MOI in MT-4 resulted in a population, designated F15(−3), depicting the SI phenotype. This phenotypic shift was reproducibly observed upon independent, repeated passages of F0.

The replication rates of the initial clones and of the passaged viruses were quantified by measuring the RT activity in the culture medium of MT-4 cells infected with a normalized amount of the relevant viruses. Populations D15(−1) and E15(−3) showed a 1-day delay in the appearance of RT activity in the culture medium relative to their respective parental clones D0 and E0. The opposite effect, that is, appearance of RT activity in advance of the parental clone, was observed with population A15(−3) (data not shown).

Clones A0 to F0 were similar to the original S61 isolate with regard to their low level replication in PBMC and monocyte/macrophage cultures and in the ability to induce syncytia in PBMC, contrary to the result for MT-4 cells. Interestingly, all viruses tested replicated 10-fold more efficiently (as determined by the RT activity assay) in the U937 clone 2 cell line than in primary cells. Clones A0, C0, D0, and F0 did not replicate in the HuT-78 cell line, a result unexpected for viral clones adapted to grow in T cells; replication of clones B0 and E0 and of the initial isolate was detectable only by using the p24 antigen test.

Serial passage in MT-4 cells resulted in only minor changes in the replicative properties of the viral clones in the other cell lines tested. For example, clone C0 did not replicate in HuT-78 cells, whereas after passage at either MOI, a low-level replication could be demonstrated only by p24 antigen ELISA. Similarly, upon passage of clone F0 at a low MOI (0.001 PFU per cell), the virus population acquired the ability to replicate in HuT-78 cells, while replication in primary monocyte/macrophage cultures became barely detectable. A decrease in the ability to replicate in the monocyte/macrophage primary cultures was also observed upon serial passage of clone E0. Less pronounced differences in the ability to replicate in HuT-78 cells were found among the clones. Other modifications in the ability to replicate in primary cultures or established cell lines were of a minor nature.

Most genetic and phenotypic changes occur upon passage of HIV-1 clonal populations at a low MOI. Of a total of 13 clear genetic or phenotypic alterations found among the passaged HIV-1 populations (Table 1), 12 occurred in the course of infections at an MOI of 0.001 PFU per cell, and only 1 (the variation in replicative ability of clone D) occurred at an MOI of 0.1 PFU per cell (Table 1). To test the significance of this bias, we have applied the binominal distribution, which yields $P = 6.4 \times 10^{-4} < <\alpha = 0.05$. Thus, the probability of obtaining by chance 12 out of 13 variants upon passage of the virus at 0.001 PFU per cell (rather than the expected 6 or 7 variants at each MOI) is lower than 0.1%. Also, use of a contingency table to adjust the observed to the expected ratio of variants yields $\chi^2 = 9.37 > \chi^2_{0.05}$, again indicating that dilute passage promoted the dominance of HIV-1 variants in a highly significant way. (If additional, minor changes in replicative ability in primary cultures or cell lines [see above] are also considered in the calculations, the strong bias in favor of expression of variations at low MOIs is maintained.) This may be explained by a number of possible mechanisms. It may be thought that since at the lower MOI used, more replication cycles occurred, then more mutations

accumulated. However, given the elevated mutation rates and frequencies in HIV-1 and the continuous action of negative selection (42), it is unlikely that the mere number of replication rounds is the main contributor to genetic diversification. It appears also unlikely that defective HIV-1 genomes can cause interference and increased variation at the lower MOI used. In fact, the two passage conditions (involving infections with less than 1 PFU per cell) were chosen to minimize the generation of defective genomes either by accumulation of point mutations or by recombination. An interesting possibility is the suppressive effect exerted by highly populated quasispecies on variants with a potential advantage included in their mutant spectra. This important observation was predicted from simulation studies on quasispecies (reviewed in reference 17) and experimentally demonstrated by de la Torre and Holland (10). They showed that a highly competitive clone of vesicular stomatitis virus could become dominant in an evolving population only when seeded above the threshold level during passage. Our results suggest that HIV-1 variants with distinct genetic and phenotypic characteristics have been kept at low, undetectable levels unless dilution diminished the suppressive effects of a populated quasispecies, as suggested by de la Torre and Holland (10). It appears that not only bottlenecking (5, 16, 47) but also constrictions and expansions of population size may be an important influence in RNA virus heterogeneity and evolution by favoring and suppressing, respectively, the dominance of variants initially hidden in the quasispecies.

Phenotypic alterations of HIV-1 in vivo are also observed in cell culture. Phenotypic modifications of HIV-1 previously identified in AIDS patients, such as syncytium-forming ability, replication level, cell tropism, and substitutions at the V3 loop of gp120, were readily observed upon passage of cloned virus in cell culture. Thus, such modifications are not necessarily the result of any particular disease stage or of immunological or physiological alterations in AIDS patients, since they occur also in the constant environment provided by a cell culture system. In particular, it is noteworthy that a change from NSI to SI phenotype was observed in the absence of any immunological selection. As previously noticed for antigenic domains of other RNA viruses such as foot-and-mouth disease virus (12, 13), rabies virus (2), influenza virus (36), or the lentivirus equine infectious anemia virus (4), amino acid substitutions were fixed at the main antigenic loop V3 of gp120 (18, 21, 28, 31, 33) upon passage of HIV-1 in the absence of antibodies. This domain has also been related to other properties such replication capacity, tropism, or CPE phenotype. The extent of genetic heterogeneity estimated considering the passaged populations analyzed (about 0.03% of the nucleotides screened, or an average of three point mutations per genome) is very similar to that found among individual clones of foot-and-mouth disease virus derived from a cloned virus population passaged in lytic or persistent infections in cell culture (9, 40). This finding suggests that the extensive divergence and heterogeneity attained by HIV-1 in vivo is probably influenced by the long periods of infection, including multiple sampling events, random activation of proviruses, and episodes of positive selection, rather than due to an increased ability of HIV-1 (compared with other riboviruses and retroviruses) to produce variants during replication.

We are indebted to A. García-Sáiz, C. Casado, and J. M. Varela for advice on HIV-1 serology, L. Pérez-Alvarez for clinical data, I. Nájera for polymerase chain reaction sequencing protocols, and S. F. Elena (University of Valencia) for help with statistical procedures. The expert technical assistance of Åsa Bjorndal is gratefully acknowledged. We thank C. Rodero and Angel del Pozo for artwork.

Work at CNBCR was supported by DGICYT grant PM88-0055, CICYT grant SAF 92-0103, institutional support from Instituto Carlos III, and a concerted action between Instituto Carlos III and Bristol-Myers Squibb Spain. Work at the CBM supported by grant PB91-0051-C02-01 from DGICYT and Fundación Ramón Areces. Work at the Department of Virology, Karolinska Institute, was supported by the U.S. Army Medical Research and Development Command under grant DAMD17-89-Z 9038 and by the Swedish National Board for Industrial and Technical Development. S.S.-P. is a recipient of a predoctoral fellowship from Instituto Carlos III.

REFERENCES

1. **Åsjö, B., U. K. Sharma, L. Morfeldt-Månson, A. Magnusson, T. Barkhem, J. Albert, E. Olausson, A. von Gegerfelt, B. Lind, P. Biberfeld, and E. M. Fenyö.** 1990. Naturally occurring HIV-1 isolates with differences in replicative capacity are distinguished by in situ hybridization of infected cells. AIDS Res. Hum. Retroviruses **6:**1177–1182.
2. **Benmansour, A., M. Brahimi, C. Tuffereau, P. Coulon, F. Lafay, and A. Flamand.** 1992. Rapid sequence evolution of street rabies glycoprotein is related to the highly heterogeneous nature of the viral population. Virology **187:**33–45.
3. **Benn, S., R. Rutledge, T. Folks, J. Gold, L. Baker, J. McCormick, P. Feorino, P. Piot, T. Quinn, and M. Martin.** 1985. Genomic heterogeneity of AIDS retroviral isolates from North America and Zaire. Science **230:**1548–1553.
4. **Carpenter, S., L. H. Evans, M. Sevoian, and B. Chensebro.** 1990. In vivo and in vitro selection of equine infectious anemia virus variants. Appl. Virol. Res. **2:**99–115.
5. **Chao, L.** 1990. Fitness of RNA virus decreased by Muller's ratchet. Nature (London) **348:**454–455.
6. **Cheng-Mayer, C., J. Homsy, L. A. Evans, and J. A. Levy.** 1988. Identification of human immunodeficiency virus subtypes with distinct patterns of sensitivity to serum neutralization. Proc. Natl. Acad. Sci. USA **85:**2815–2819.
7. **Coffin, J. M.** 1986. Genetic variation in AIDS viruses. Cell **46:**1–4.
8. **Coffin, J. M.** 1992. Genetic diversity and evolution of retroviruses. Curr. Top. Microbiol. Immunol. **176:**143–164.
9. **Delassus, S., R. Cheynier, and S. Wain-Hobson.** 1992. Inhomogeneous distribution of human immunodeficiency virus type 1 proviruses within spleen. J. Virol. **66:**5642–5645.
10. **de la Torre, J. C., and J. J. Holland.** 1990. RNA virus quasispecies populations can suppress vastly superior mutant progeny. J. Virol. **64:**6278–6281.
11. **de la Torre, J. C., E. Martínez-Salas, J. Díez, A. Villaverde, F. Gebauer, E. Rocha, M. Dávila, and E. Domingo.** 1988. Coevolution of cells and viruses in a persistent infection of foot-and-mouth disease virus in cell culture. J. Virol. **62:**2050–2058.
12. **Díez, J., M. Dávila, C. Escarmís, M. G. Mateu, J. Domínguez, J. J. Pérez, E. Giralt, J. A. Melero, and E. Domingo.** 1990. Unique amino acid substitutions in the capsid proteins of foot-and-mouth disease virus from a persistent infection in cell culture. J. Virol. **64:**5519–5528.
13. **Domingo, E., C. Escarmís, M. A. Martínez, E. Martínez-Salas, and M. G. Mateu.** 1992. Foot-and-mouth disease virus populations are quasispecies. Curr. Top. Microbiol. Immunol. **176:**33–47.
14. **Domingo, E., and J. J. Holland.** 1988. High error rates, population equilibrium of RNA replication systems, p. 3–36. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics, vol. 3. CRC Press Inc., Boca Raton, Fla.
15. **Domingo, E., D. Sabo, T. Taniguchi, and C. Weissmann.** 1978. Nucleotide sequence heterogeneity of an RNA phage population. Cell **13:**735–744.
16. **Duarte, E., D. Clarke, E. Domingo, and J. Holland.** 1992. Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet. Proc. Natl. Acad. Sci. USA **89:**6015–6019.
17. **Eigen, M., and C. Biebricher.** 1988. Sequence space and qua-

sispecies distribution, p. 211–245. *In* E. Domingo, J. J. Holland, and P. Ahlquist (ed.), RNA genetics, vol. 3. CRC Press Inc., Boca Raton, Fla.

18. **Emini, E. A., W. A. Schleif, J. H. Nunberg, A. J. Conley, Y. Eda, S. Tokiyoshi, S. D. Putney, S. Matsushita, K. E. Cobb, C. M. Jett, J. W. Eichberg, and K. K. Murthy.** 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature (London) **355:**728–730.
19. **Fenyö, E. M., J. Albert, and B. Åsjö.** 1989. Replicative capacity, cytopathic effect and cell tropism of HIV. AIDS 3(Suppl. 1):s5–s12.
20. **Fenyö, E. M., L. Morfeld-Manson, F. Chiodi, B. Lind, A. von Gegerfelt, J. Albert, E. Olausson, and B. Åsjö.** 1988. Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates. J. Virol. **62:**4414–4419.
21. **Goudsmit, J., C. Debouck, R. H. Meloen, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and D. C. Gajdusek.** 1988. Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees. Proc. Natl. Acad. Sci. USA **85:**4478–4482.
22. **Groenink, M., R. A. M. Fouchier, R. E. Y. De Goede, F. De Wolf, R. A. Gruters, H. T. M. Cuypers, H. G. Huisman, and M. Tersmette.** 1991. Phenotypic heterogeneity in a panel of infectious molecular human immunodeficiency virus type 1 clones derived from a single individual. J. Virol. **65:**1968–1975.
23. **Hahn, B. H., G. M. Shaw, M. E. Taylor, R. R. Redfield, P. D. Markham, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, E. S. Parks, and W. P. Parks.** 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. Science **232:**1548–1553.
24. **Harada, S., Y. Koyanagi, and N. Yamamoto.** 1985. Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay. Science **229:**563–566.
25. **Holland, J. J., J. C. de la Torre, and D. A. Steinhauer.** 1992. RNA virus populations as quasispecies. Curr. Top. Microbiol. Immunol. **176:**1–20.
26. **Holland, J. J., K. Spindler, F. Horodyski, E. Grabau, S. Nichol, and S. VandePol.** 1982. Rapid evolution of RNA genomes. Science **215:**1577–1585.
27. **Jansen, R. W., G. Siegl, and S. Lemon.** 1990. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proc. Natl. Acad. Sci. USA **87:**2867–2871.
28. **Javaherian, K., A. J. Langlois, C. McDanal, K. L. Ross, L. I. Eckler, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1989. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. Proc. Natl. Acad. Sci. USA **86:**6768–6772.
29. **López-Galíndez, C., J. A. López, J. A. Melero, L. de la Fuente, C. Martínez, J. Ortín, and M. Perucho.** 1988. Analysis of genetic variability and mapping of point mutations in influenza virus by RNAse A mismatch cleavage method. Proc. Natl. Acad. USA **85:**3522–3526.
30. **López-Galíndez, C., J. M. Rojas, R. Nájera, D. D. Richman, and M. Perucho.** 1991. Characterization of genetic variation and 3′-azido-3′-deoxythymidine-resistance mutations of human immunodeficiency virus by the RNase A mismatch cleavage method. Proc. Natl. Acad. USA **88:**4280–4284.
31. **Meloen, R. H., R. M. Liskamp, and J. Goudsmit.** 1989. Specifity and function of the individual amino acids of an important determinant of human immunodeficiency virus type 1 that induces neutralizing activity. J. Gen. Virol. **70:**1505–1512.
32. **Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Manson, B. Åsjö, and S. Wain-Hobson.** 1989. Temporal fluctuations in HIV quasispecies *in vivo* are not reflected by sequential HIV isolations. Cell **58:**901–910.
33. **Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Hayness.** 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. Proc. Natl. Acad. Sci. USA **85:**1932–1936.
34. **Perucho, M.** 1989. Detection of single-base substitutions with the RNase A mismatch cleavage method. Strategies **2:**37–41.
35. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
36. **Rocha, E., N. J. Cox, R. A. Black, M. W. Harmon, C. J. Harrison, and A. P. Kendal.** 1991. Antigenic and genetic variation in influenza A (H1 N1) virus isolates recovered from a persistently infected immunodeficient child. J. Virol. **65:**2340–2350.
37. **Ruano, G., and K. K. Kidd.** 1991. Coupled amplification and sequencing of genomic DNA. Proc. Natl. Acad. Sci. USA **88:**2815–2819.
38. **Saag, M. S., B. H. Hahn, J. Gibbons, Y. Li, E. S. Parks, W. P. Parks, and G. M. Shaw.** 1988. Extensive variation of human immunodeficiency virus type 1 *in vivo*. Nature (London) **334:**440–444.
39. **Shaw, G. M., B. H. Hahn, S. K. Arya, J. E. Groopman, R. C. Gallo, and F. Wong-Staal.** 1984. Molecular characterization of human T-cell leukemia (lymphotropic) virus type III in the acquired immune deficiency syndrome. Science **226:**1165–1171.
40. **Sobrino, F., M. Dávila, J. Ortín, and E. Domingo.** 1983. Multiple genetic variants arise in the course of replication of foot-and-mouth disease virus in cell cultures. Virology **128:**310–318.
41. **Sundqvist, V. A., J. Albert, E. Ohlsson, J. Hinkula, E. M. Fenyö, and B. Wahren.** 1989. Human immunodeficiency virus type 1 p24 production and antigenic variation in tissue culture of isolates with various growth characteristics. J. Med. Virol. **29:**170–175.
42. **Temin, H. M.** 1989. Is HIV unique or merely different? J. Acquired Immune Defic. Syndr. **2:**1–9.
43. **Tersmette, M., R. A. Gruters, F. de Wolf, R. E. Y. de Goede, J. M. A. Lange, P. T. H. Schellekens, J. Goudsmit, J. G. Huisman, and F. Miedema.** 1989. Evidence for a role of virulent human immunodeficiency virus (HIV) variants in the pathogenesis of acquired immunodeficiency syndrome: studies on sequential HIV isolates. J. Virol. **63:**2118–2125.
44. **Valentin, A., J. Albert, E. M. Fenyö, and B. Åsjö.** 1990. HIV-1 infection of normal human macrophage cultures: implications for silent infection. Virology **177:**790–794.
45. **Wain-Hobson, S.** 1992. Human immunodeficiency virus type 1 quasispecies *in vivo* and *ex vivo*. Curr. Top. Microbiol. Immunol. **176:**181–193.
46. **Willey, R. L., D. H. Smith, L. A. Lasky, T. S. Theodore, P. L. Earl, B. Moss, D. J. Capon, and M. A. Martín.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is critical for infectivity. J. Virol. **62:**139–147.
47. **Wolinsky, S. M., C. M. Wike, B. T. Korber, C. Hutto, W. P. Parks, L. L. Rosenblum, K. J. Kunstman, M. R. Furtado, and J. L. Muñoz.** 1992. Selective transmission of human immunodeficiency virus type 1 variants from mothers to infants. Science **235:**1134–1137.
48. **Wong-Staal, F., G. M. Shaw, B. H. Hahn, S. Z. Salahuddin, M. Popovic, P. Markham, R. Redfield, and R. C. Gallo.** 1985. Genomic diversity of human T-lymphotropic virus type III (HTLV-III). Science **229:**759–762.