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Human immunodeficiency virus type 1 population bottleneck during indinavir therapy causes a genetic drift in the *env* quasispecies

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The impact of emergence of genetic resistance, soon after the beginning of antiretroviral therapy, on the genotype of other viral loci not implicated in the development of resistance was studied in four human immunodeficiency type 1 (HIV-1)-infected patients subjected to indinavir monotherapy. Two patients were chosen because they showed no decrease in virus load during the study period and two were selected because they showed a rapid decline in plasma viraemia after the initiation of therapy and a virus rebound after 12 weeks of treatment. The evolution of virus sequences was analysed within the four infected patients by examining virus sequences spanning the protease and C2–V3 *env* genes by RT–PCR of plasma samples obtained at the beginning and after 12 weeks of therapy. PCR products from the two genomic regions from the two sample points per patient were cloned and 10–15 clones from each sample were sequenced. Genotypic indinavir resistance was present in the four patients after 12 weeks of therapy. The overall protease and C2–V3 *env* regions quasispecies diversity at time zero was higher than that after 12 weeks of therapy, but this difference was more significant in the two patients who showed a reduction in virus load soon after the initiation of treatment. C2–V3 *env* sequences indicated that changes during emergence of resistance to indinavir were only detected in the two patients who showed a drastic reduction in virus load. Thus, a temporal relationship was observed between the start of therapy, a drastic reduction in virus load and a drift in the HIV-1 *env* quasispecies.

Introduction

The introduction of antiretroviral monotherapy demonstrated a delay in the disease progression and prolonged survival in human immunodeficiency virus type 1 (HIV-1)-infected patients. Nevertheless, the systematic selection of HIV-1 mutants resistant to antiretroviral inhibitors (Domingo *et al.*, 1997; Richman, 1996; Hirsch *et al.*, 1998; Shafer *et al.*, 1998; Cabana *et al.*, 1999), generally associated with a rapid rebound of patient virus loads, has greatly limited the efficacy of these treatments until the recent encouraging results obtained with combination therapy (Hammer *et al.*, 1997; Gulick *et al.*, 1997). Virus resistance is a direct consequence of genetic diversity. The high mutation rate of HIV-1 reverse transcriptase (Mansky & Temin, 1995) and the high replication levels of virus in an infected patient (Ho *et al.*, 1995; Wei *et al.*, 1995) allow for the generation of many variants. Distinct viral genomes arise continuously in any infected patient, forming a complex 'swarm' of related genomes termed quasispecies (Domingo *et al.*, 1996). Thus, when drug pressure is applied, resistant viruses can emerge within weeks because of the prior existence of drug-resistant mutants (Nájera *et al.*, 1995; Lech *et al.*, 1996; Havlir *et al.*, 1996). We hypothesize that the population bottleneck produced during antiviral therapy might randomly select changes in other genomic regions not directly implicated in the generation of drug resistance. Virus genetic bottlenecks are important because bottlenecked populations will often have a genetic composition different from that of the parental population (Chao, 1990; Duarte *et al.*, 1992; Clarke *et al.*, 1993; Novella *et al.*, 1995; Escarmis *et al.*, 1996). To determine and quantify the effect that a strong selective pressure on the viral protease has on the genetic variation of an unrelated gene, we have analysed the quasispecies evolution in the *env* and *pol* genes soon after the beginning of indinavir monotherapy.

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The GenBank accession numbers of the HIV-1 sequences described in this study are AF142154–AF142239 (protease) and AF142240–AF142320 and AF175569–AF175573 (*env*).

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Here we report the genomic fluctuations in HIV-1 populations from patients subjected to indinavir monotherapy. We analysed the virus sequence evolution of the protease and C2–V3 *env* gene regions in four infected patients. Viral RNA from plasma samples obtained at the beginning of treatment and after 12 weeks of drug therapy was amplified by RT–PCR. Products were cloned and 10–15 clones from each sample were sequenced. A neighbour-joining phylogenetic analysis of the sequences from the patients who were transient responders showed that the *env* and protease sequences at the beginning of treatment clustered distinctly from sequences obtained after therapy. Such changes in quasispecies were not detected in patients in whom population bottlenecks were not observed, that is, in the non-responders. These results document that population bottlenecks during indinavir therapy can cause genetic modifications not only in the protease gene but also in other genomic regions, in particular in the *env* quasispecies.

Methods

Patients. Four patients with $CD4^+$ cell counts below 100 cells/µl (median 30 cells/µl) were selected from an indinavir monotherapy trial (Ruiz *et al.*, 1998). At the beginning of the study, the 25 patients included in this trial had a median $CD4^+$ count of 20 cells/µl (range 0–80 cells/µl) and a median plasma HIV-1 RNA level of 5·4 $\log_{10}$/µl (range 3·6–6·7 $\log_{10}$/µl). Patients A and B were included in the present study because they showed no decrease in plasma HIV-1 RNA level during the study period (Fig. 1). Patients C and D were selected because they showed a decrease of 2 $\log_{10}$ in plasma HIV-1 RNA level within 2 weeks of the start of treatment (Fig. 1). The four patients selected received 800 mg indinavir every 8 h for 24 weeks. Patients A to D had previously been treated with one, two or three dideoxynucleoside analogue inhibitors (AZT, ddC, ddI, 3TC) for at least 6 months. Plasma virus load was determined in these four patients by using the amplicor assay (Roche Molecular Systems).

Recovery and amplification of plasma viral RNA. RNA was extracted from 140 µl plasma as described by Boom *et al.* (1990), based on a guanidinium isothiocyanate lysis buffer and glass-milk, and resuspended in 50 µl TE buffer. After isolation of viral RNA, 10 µl resuspended RNA (corresponding to 28 µl plasma) was reverse-transcribed and amplified by using the Titan one-tube RT–PCR system (Boehringer Mannheim) according to the manufacturer's instructions. Briefly, the RT–PCR mixture contained 10 pmol of protease oligonucleotides 5′prot 1 (sense) (5′ AGGCTAATTTTTTAGGGAAGATCTGGCCTTCC 3′, HXB2 positions 2077–2108) and 3′prot 1 (antisense) (5′ GCACCTACTGGAGTATTGTATGGATTTTCAGG 3′, 2703–2733) or C2–V3 *env* oligonucleotides ARP 825 (sense) (5′ GCACAGTACAATGTACACAT 3′, 6951–6970) and ARP 829 (5′ CAATTAAAACTGTGCGTTAG 3′,

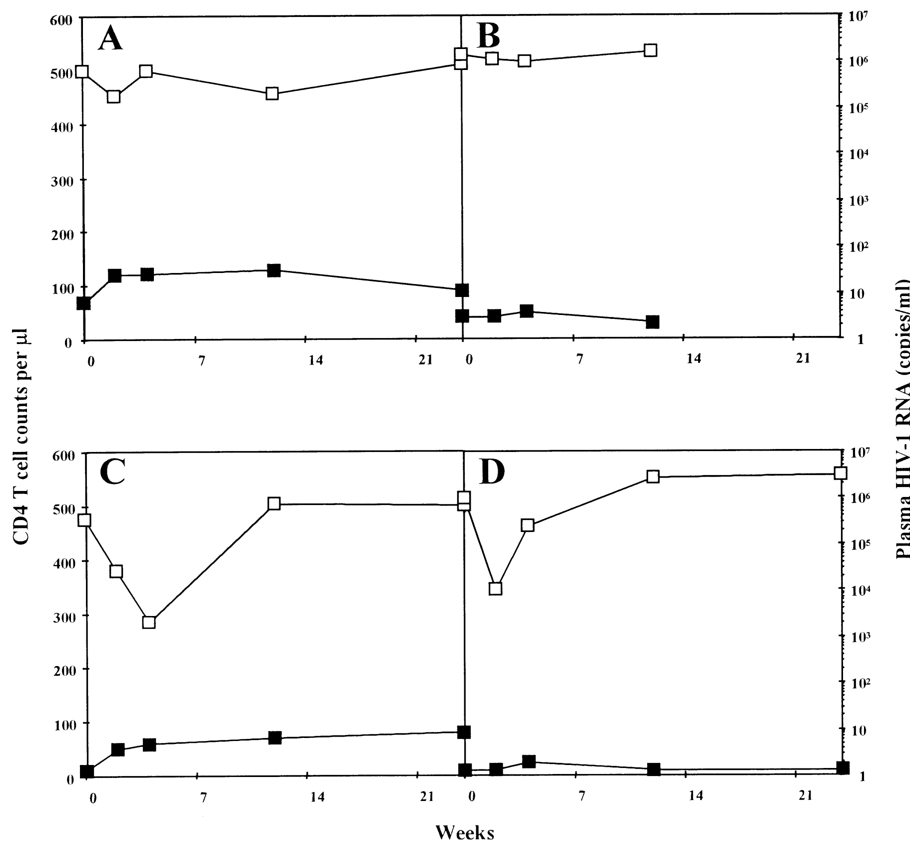

Fig. 1. Summary of quantitative HIV-1 virion-associated RNA and $CD4^+$ T cell counts for each of the four infected patients (A–D). The time after the beginning of therapy is shown on the *x*-axis. $CD4^+$ T cell counts were measured by flow-activated cytometric assay and expressed per µl blood (■). HIV-1 RNA levels were measured by a quantitative RT–PCR assay and are expressed in copy number per ml plasma (□). Sequencing was performed at time zero and after 12 weeks of indinavir therapy.

7337–7356), 200 μM dNTPs, 1·5 mM $MgCl_2$, RT–PCR buffer, 5 mM DTT, 5 U RNase inhibitor and 1 μl enzyme mixture (AMV and Expand High Fidelity PCR system) in a total volume of 50 μl. The samples were incubated as follows: 30 min at 50 °C; one cycle of denaturation at 94 °C for 2 min; 10 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 68 °C for 1 min; and then 25 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 68 °C for 1 min with 5 s added for each cycle. A final extension at 68 °C for 7 min was added to the last cycle.

A 5 μl aliquot from the first PCR was amplified in a 100 μl reaction mixture containing 10 pmol of protease oligonucleotides 5′prot 2 (sense) (5′ TCAGAGCAGACCAGAGCCAACAGCCCCCA 3′, HXB2 positions 2135–2162) and 3′prot 2 (antisense) (5′ GCAAATACTGGAGTATTGTATGGATTTTCAGG 3′, 2770–2792) or C2–V3 *env* oligonucleotides ARP 826 (sense) (5′ CGCTAGGAATTCGGCCAGTAGTATCAACTCAA 3′, 7013–7029) and ARP 828 (antisense) (5′ GTACACAAGCTTTCTGGGTCCCCTCCTGAGGA 3′, 7313–7334), 200 μM dNTPs, 1·5 mM $MgCl_2$, PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8·3) and 0·5 U *Taq* DNA polymerase (Perkin-Elmer). Cycling parameters were one cycle of denaturation at 94 °C for 2 min and then 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. This was followed by a 7 min incubation at 72 °C. All PCRs were run with negative controls and employed procedures to prevent sample contamination. The sensitivity of the above nested PCR was determined to be one copy, based on nested PCR of a dilution series of an HIV-1 control DNA (HIVZ6) accurately titrated for copy number (Perkin-Elmer). The amount of input cDNA for the first step of the nested PCR was determined by PCR amplification of serial dilutions of the plasma viral RNA. An input cDNA copy number of 20 times the end-point for positive PCR amplification was used to ensure that multiple HIV templates were present in each sample.

■ **Cloning and sequencing.** To verify protease and C2–V3 *env* gene amplification and to estimate product yield, 5% of the nested PCR mixture was run on a 1·5% agarose gel. PCR products were purified by using the Qiaquick spin PCR purification kit (Qiagen). Approximately 50 ng DNA was ligated with the TA cloning plasmid pGEM T (Promega). Competent *Escherichia coli* XL-1 cells were then transformed and screened for white colonies on ampicillin–IPTG–X-Gal agar plates. A small-scale plasmid preparation was carried out to recover bacterial DNA. Isolated plasmid DNA was screened for protease or C2–V3 *env* genes by PCR with oligonucleotides 5′prot 2 and 3′prot 2 or ARP 826 and ARP 828, respectively. For each sample, DNA from about 10 colonies was sequenced with the ABI PRISM dRhodamine terminator cycle sequencing kit (Applied Biosystems). The products of the reactions were then analysed on an Applied Biosystems 310 sequencer. Sequencing oligonucleotides for the protease gene were 5′prot 2 and 3′prot 2, and ARP 826 and ARP 828 for the C2–V3 *env* gene. Sequence editing was performed by using the Sequence Navigator program (Applied Biosystems).

■ **Analysis of the sequence data.** Sequences were aligned by using CLUSTAL W (Thompson *et al*., 1994). Phylogenetic reconstructions were generated by using the neighbour-joining method in the PHYLIP software (Felsenstein, 1988, 1995), with a maximum-likelihood distance matrix and a ratio of transition to transversion of 2·0 (programs DNADIST and NEIGHBOR). Bootstrap resampling (Felsenstein, 1985) was applied to the neighbour-joining trees (programs SEQBOOT and CONSENSE) to assign approximate confidence limits to individual branches. The final graphical output was created with the program TreeView (Page, 1996). The proportions of synonymous substitutions per potential synonymous site and nonsynonymous substitutions per potential nonsynonymous site were calculated with the program WET (Dopazo, 1995). The distributions of DNA distances for each time-point and patient were subjected to nonparametric statistical treatment by using Wilcoxon's signed rank test included in the SPSS version 7.5 software package (SPSS Inc.).

Results

Virological characterization of the patients

Two of the four patients chosen for this study and termed transient-responders (patients C and D) displayed a drastic drop in their plasma viraemia of more than 2 logs after 2–4 weeks of indinavir therapy, with a rebound to time-zero values after 12 weeks (Fig. 1). The other two patients (A and B) showed no decrease in their virus load during the 24 weeks of follow-up, and consequently were considered non-responders. Plasma concentrations of indinavir were measured for the four patients at the beginning of therapy and at 2, 4, 12 and 24 weeks of therapy to rule out the possibility that virus rebound after therapy was due to patient non-compliance (Ruiz *et al*., 1998). The rationale for including non-responders and transient-responders in this study was to elucidate whether the virus bottleneck generated soon after indinavir therapy influenced evolution of the *env* gene.

Quasispecies changes in the protease gene after indinavir therapy

Fig. 2(*a*) shows an alignment of the deduced amino acid sequences of the protease nucleotide sequences obtained for patients A to D at the beginning of the therapy and at 12 weeks of therapy. After the start of therapy, the four patients presented clones with substitutions at critical positions, residues 46 and 82, in the development of indinavir resistance (Boden & Markowitz, 1998). Secondary substitutions in the development of indinavir resistance, residues 10, 32, 63, 71 and 90, were also detected in the four patients, but no two presented the same pattern of substitutions. Some of these secondary substitutions were observed before the introduction of the therapy, at residues 10 (patient C), 63 (A, B, C and D), 71 (B) and 90 (A). In contrast, no critical substitutions were observed before the treatment. No clear differences in the development of genetic resistance were detected between transient and non-responder patients. In addition, after 24 weeks of treatment (12 for patient B), the four patients presented phenotypic resistance to indinavir (Table 1) (Ruiz *et al*., 1998).

Diversity in the protease sequence was evaluated quantitatively for each patient by computing the inter- and intra-time-point genetic distances derived from individual protease molecular clones from both the initial and final time-points. The rate of quasispecies evolution was derived for each patient by analysis of the inter-time-point distances. The means $\pm$ SD

(*a*)

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                     *        20         *        40         *        60         *        80         *       99
ConsensusB: PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMNLPGRWKPKMIGGIGGFIKVRQYDQILIEICGHKAIGTVLVGPTPVNIIGRNLLTQIGCTLNF :  99
Awk 0 10  : ..................................D.A.........................P.................................... :  99 x6
Awk 0 12  : ..................................D.A.........................P..........................M......... :  99 x3
Awk 0  6  : ..................................D.T.........................P......................R............. :  99

Awk12 37  : ..................................D.A.........................P..................A................. :  99 x3
Awk12 26  : ..................................D.A.........................P.................................... :  99 x2
Awk12 41  : ..................................D.A.........................P..........................M......... :  99 x2
Awk12 44  : ..................................D.A........L................P.......T..........A.......S.....S... :  99 x2
Awk12 38  : ..................................D.A.........................P..............R...A................. :  99
Awk12 30  : ....................................A.........................P..................T................. :  99

Bwk 0  1  : ..................................D...S......................VP.......V.....I...............L...... :  99 x4
Bwk 0  5  : ..................................D...S......................VP.......V.....I...............L...... :  99 x2
Bwk 0 13  : ............M.........................S......................VP.......V.....I...............L...... :  99
Bwk 0  2  : ..................................D...S....................G.VP.......V.....I...............L...... :  99
Bwk 0  6  : ......................................S......................VP.......V.....I...............L...... :  99
Bwk 0 67  : ..................................D...S........R.............VP.......V.....I...............L...... :  99

Bwk12 18  : ......................................S......................VP.......V.S...I............M..L...... :  99 x3
Bwk12 24  : ......................................S......................VP.......V.S...I............M..L...... :  99
Bwk12 25  : .........P............................S......................VP.......V.S...I............M..L...... :  99
Bwk12 26  : ......................................S......................VP.......V.S...I.........K..M..L...... :  99
Bwk12 27  : ..................................D...S..................E...VP.......V.....I.......V....M..L...... :  99
Bwk12 74  : ......................................S......................VP.......V.....I............M..L...... :  99
Bwk12 75  : ....P.................................S......................VP.......V.S...I.........K..M..L...... :  99
Bwk12 79  : ......................................S......................VP.......V.S...I....A.......M..L...... :  99

Cwk 0 50  : ...........PVR.................................................P..................I................. :  99 x3
Cwk 0 49  : .............M....R............................................P.................................... :  99
Cwk 0 52  : ......P....PV.....................D............................P.................................... :  99
Cwk 0 55  : ...........P.R....................D............................P.................................... :  99
Cwk 0 56  : .........I..V..................................................P..................I................. :  99
Cwk 0 57  : ...........PVR.....................I...........R.........L....P..................I................. :  99
Cwk 0 60  : .............R....................D.D....................L....P.....R.............................. :  99
Cwk 0 51  : .............M....R............................................P......T............................. :  99

Cwk12 66  : ........SI..V..................................................P............V............I.......... :  99 x2
Cwk12 73  : .........I..V..................................................P............V............I.......... :  99 x2
Cwk12 74  : .........I..V................................L.................P............V............I.......... :  99 x2
Cwk12 65  : ........SI..V.....................D...........L...F.............P..................A......I.......... :  99
Cwk12 67  : .........I..V.........................R........................P............V............I.......... :  99
Cwk12 70  : .........I..V.M........................................A......P............V............I.......... :  99
Cwk12 64  : ........SI..V...........................................K.....P............V............I.......... :  99

Dwk 0  1  : ..............VE......................S....................E..P.................................... :  99 x7
Dwk 0  6  : ..............VE......................S.K..................E..P................................R.... :  99 x2
Dwk 0  5  : ..............VE...........V..........S....................E..P.................................... :  99
Dwk 0  7  : ..............VE...................I..S.K..................E..P.................................... :  99
Dwk 0  9  : ..............VE......................S.K..................E..P.................................... :  99
Dwk 0 11  : ..............VE......................S....................E.VP.................................... :  99

Dwk12 14  : ..............VE...............I....K.S......I.............E..P......EV..........A...............K. :  99 x6
Dwk12 12  : ..............VE...............I......S......I.............E..P.......V............................ :  98
Dwk12 13  : ..............VE...............I......S......I.............E..P...R...V..........A................. :  99
Dwk12 13r : ..............VE...............I......S......I.............E..P...R...V..........A........N........ :  99
Dwk12  2  : ..............VE...............I......S......I.............E..P.......V..........A................. :  99
Dwk12  3  : ..............VE......................S......I.............E..P.......V..........A................. :  99
Dwk12  4  : ..............VE...............I......S......I.............E..P.......V..........A..........T...... :  99
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Fig. 2. For legend see facing page.

of these distances for each patient are given in Table 2. Transient-responder patients C and D showed a higher rate of quasispecies evolution compared with the non-responder patients A and B (Table 2). Taken as subcohorts, the rate of quasispecies evolution in the transient-responder patients ($3{\cdot}3 \pm 0{\cdot}8$) was higher than that for the non-responder patients ($1{\cdot}9 \pm 1{\cdot}1$) ($P < 0{\cdot}001$; Wilcoxon's signed rank test). Transient-responder patients C and D showed a statistically significant decrease in DNA sequence variation after therapy ($P < 0{\cdot}001$ and $P < 0{\cdot}001$, respectively) (Table 3). In contrast, non-responder patients A and B showed no significant change in DNA diversity during this 12 week period ($P = 0{\cdot}835$ and $0{\cdot}651$, respectively). Consequently, a significant decrease in quasispecies diversity was observed only in those patients who showed a population bottleneck after the introduction of the indinavir therapy.

Selection pressure on HIV-1 quasispecies was determined by analysing the synonymous (silent) and nonsynonymous (amino acid-changing) nucleotide substitution patterns. Dominance of the rate of nonsynonymous nucleotide substitutions over the rate of synonymous substitutions is evidence for positive selection. The mean values of nucleotide substitutions per synonymous site, ds, and nonsynonymous site, dn, for all pairwise sequence comparisons at both time-points were calculated (Table 3). The high ds/dn ratios observed before the therapy in the four patients indicate no selection (neutrality) at

(*b*)

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          245    *       260         *       280         *       300         *       320         *
ConsensusB: LAEEEVVIRSENFTDNAKTIIVQL?ESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNNTLKQIV?KLREQFGN : 90
Awk 0 10  : ......I......SN.......L.NKT.T...I..G................A..D...N.........STN.T.......G........ : 90
Awk 0 11  : ......I......SN.........NKT.T...I..G................A..D...N.........STNRT.......G........ : 90
Awk 0 12  : .............SN.........NKT.T...I..G........M.....Y.A..DL..N.........KTN.T...E...G....L... : 90
Awk 0  4  : .............SN.........NKT.T...I..G................A..D...N.........STN.T.......G........ : 90
Awk 0  1  : ..K..........SN.........NQT.T...I..G................A..D...N.........STN.T.......GT....... : 90
Awk 0  5  : ..D..........PN.....M...NKT.T...I..G................A..D...N.........STN.T.......G........ : 90
Awk 0  6  : .............SN.........NKT.T...I..G................A..D...N..........TN.T...E...G........ : 90
Awk 0  7  : ......A......SN.........SKT.T...I..G................A..D...N.........STN.T.......G........ : 90
Awk 0  8  : ......I......SN.........NRT.T...I..G................A..D...N..........TD.T.......S........ : 90
Awk 0  9  : .............S..........NKT.T.S....G................A..D...N.........STN.T.......G........ : 90

Awk12 24  : ......I......SN.........NRT.T...I..G................A..D...N..........TD.T.......G........ : 90 x2
Awk12  1  : .............SN.........NKT.T...I..G................A..D...N..........TD.T.......G........ : 90
Awk12 26  : ......I......SN.........NKT.T...I..G................A..D...N.........STN.T.......G........ : 90
Awk12 27  : .............SN.......L.NKT.T...I..G................A..D...N..........TN.T...E...G........ : 90
Awk12 29  : .............SN.........NKT.T...I..G................A..D...N..........TN.T.......G........ : 90
Awk12 31  : .............SN.........NKT.T...I..G................A..D...N..........TN.T...E...G........ : 90

Bwk 0  1  : .............S..........NK.I...........SE.......K...A.KR......K.Y.....VQ..E......G......E. : 90 x5
Bwk 0  2  : .............S..........NK.I...........SD.......K...A.KR......K.Y.....VQ..E......E......E. : 90
Bwk 0  3  : .............S...R......NK.I...........SE.......K...A.KR......K.Y.....VQ..E......G......E. : 90
Bwk 0  5  : ......I......S..........NK.I...........SD.......K...A.KR.....KK.Y.....VQ..E......G......E. : 90
Bwk 0  9  : .............S..........NK.I...........SE.......K.S.A.KR......KSY.....VQ..E......G......E. : 90

Bwk12  1  : .............S..........NK.I...........SE.......K...A.KR......K.Y....KVQ..E......G......E. : 90 x11
Bwk12 21  : .............S..........NK.I...........SE.......K...A.KR......K.Y.....VQ..E......G......E. : 90 x3
Bwk12  7  : .............S..........NK.I.........S.SE.......K...A.KR......K.Y....KVQ..E......G......E. : 90

Cwk 0 12  : ..........Q.............K.A........G.......PV.....I....T.............K....S......K..Q..... : 90 x6
Cwk 0 16  : ..........Q......R......K.A........G.......PV.....I....T.............K....S......K..Q..... : 90
Cwk 0 17  : ..........Q.............K.A........S....N..QL.....I......................DS.....AK...V.... : 90
Cwk 0 41  : ..........K.............K.A........G.......PV.....I....A..................S..G...K........ : 90
Cwk 0 42  : ..........Q.L....R......K.A........G....N...V.....I....N.................DS....VAK..K...E. : 90
Cwk 0 11  : .....I....Q.............K.A.K......G....N..PV...K.I....NM................DS....VAK..K...D. : 90

Cwk12 33  : ..........Q.............K.A........G....N...V...K.I....N.................SS.....AK..K...E. : 90 x7
Cwk12 31  : ..........Q.............K.A.K......G....N...V...K.I....N.................SS.....AK..K...E. : 90
Cwk12 39  : ..........Q.............K.A........GS...N...V...K.I....N.................SS.....AK..K...E. : 90
Cwk12 63  : ..........Q.............K.A........G....N...V.....I....N.................DS....VAK..K...E. : 90
Cwk12 64  : .....A....Q.............K.A........G....N..PV...K.I....N.................SS.....AK......E. : 90
Cwk12 65  : ..........Q......R......K.A........G....N...V.....I....N.................SS.....AK......E. : 90
Cwk12 36  : ..........Q.............K.A........G....N...V...K.I....N.................S.....VAK..K...E. : 90

Dwk 0 12  : .....I........N.........N...V............G....V...L...--.V........ILNGTH.K...R..AI..K..YNT : 88 x2
Dwk 0101  : .....I........N.........N...V............G....V...L...--.V........ILNGTH.K...R..AI..K...NT : 88 x2
Dwk 0102  : .....I........N.........N...V......S..I.RD....G...LS..--...N......SLNGTQ.....RR.AI......NI : 88 x2
Dwk 0 14  : .....I........N.........N...V......S..I.RD....G...LS..--...N......SLNGTQ.....RR.AI......NI : 88
Dwk 0 20  : .....I........N.........N..--------S..I.RD....G...LS..--...N......SLNGTQL....RR.AI......NI : 80
Dwk 0 78  : .....I........N.........N..IV..........K.G....V...L...--..........ILNG...K...R..AI..K...NI : 88
Dwk 0105  : .....I........N.........N...V............G....V...L...--.V........SLNGTH.K...R..AI..K..YNT : 88

Dwk12  1  : .....I...T....N.........N...V............G....V.G.L...--.V........ILNGTQ.....R..GI..K...NI : 88 x6
Dwk12  7  : .....I...T....N.........N...V............G....V.G.L..L--.V........ILNGTQ.....R..GI..K...NI : 88
Dwk12  8  : .....I...T....N.........N...V............G....V.G.L...--VV........ILNGTH.K...R..GI..K..YNT : 88
Dwk12  9  : .....I...T....N.........N.A.V............G....V.G.L...--.V........ILNGTQ.....R..AI..K...NI : 88
Dwk12 10  : .....I...T....N.........N...V............G....V.G.L...--.V........ILNGTQ.....R..GI..K...NI : 88
Dwk12102  : .....I........N.........N...V............G....V.G.L...--.V........ILNGTQ.....R..GI..K...NI : 88
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Fig. 2. Deduced amino acid sequence alignments of the protease (*a*) and C2–V3 regions of gp120 (*b*). The single-letter amino acid code is used. Amino acid changes are indicated relative to the HIV-1 consensus B sequence (Myers *et al.*, 1996). Protease viral sequences start at position 1 of the HIV-1 consensus B protease sequence. Envelope viral sequences start at position 245 of the *env* HIV-1 consensus B sequence (Myers *et al.*, 1996). The deduced amino acid sequences are annotated by a capital letter for each patient (A–D). The time in weeks when sequencing was performed is also shown for each sequence. The number of occurrences within each sample of identical amino acid sequences is given on the right. Dots indicate amino acid sequence identity and dashes indicate deletions.

this locus. As expected, a statistically significant decrease in the ds/dn ratios was observed in the four patients after 12 weeks of therapy. (Table 2). These lower ds/dn ratios detected after therapy demonstrate the strong positive selection exerted by the drug on the protease gene during this period.

To construct evolutionary relationships among the protease sequences obtained before and after the introduction of therapy, a neighbour-joining phylogenetic analysis of all protease sequences was carried out (Fig. 3 *a*). Interestingly, two different topological patterns were observed in the phylogenetic reconstruction of sequences from the transient-responders and the non-responders. For the transient-responders, who had a drastic drop in plasma viraemia early after the introduction of the therapy (Fig. 1), the protease sequences obtained after 12 weeks of therapy showed distinctive clustering with respect to the sequences obtained at

Table 1. Phenotypic protease resistance of virus derived from patients A to D after 24 weeks of indinavir therapy

Data were obtained from Ruiz *et al.* (1998).

Patient	Indinavir IC_{50} (μM) (-fold resistance)
A	0·147 (9)
B	0·170* (9)
C	0·092 (4)
D	0·379 (19)

* IC_{50} after 12 weeks of therapy.

Table 2. Inter-time-point distances for protease and *env* DNA sequences

DNA distances are shown as means ± SD. NR, Non-responder; TR, transient responder.

Patient	Protease	*env*
A (NR)	1·9 ± 1·3	2·1 ± 0·9
B (NR)	2·0 ± 0·6	1·0 ± 0·5
C (TR)	3·1 ± 0·9	5·2 ± 1·1
D (TR)	3·2 ± 0·8	4·5 ± 2·3
All NR	1·9 ± 1·1 ($P < 0·001$)*	1·5 ± 0·8 ($P < 0·001$)*
All TR	3·3 ± 0·8	4·9 ± 1·8

* Probability obtained by Wilcoxon's signed rank test; all NR compared with all TR.

the beginning of treatment (Fig. 3 *a*). This observation was supported by bootstrap proportions of greater than 50 of 100 bootstrap replicates, as shown in the neighbour-joining phylogenetic reconstruction. In contrast, the phylogenetic reconstruction for the non-responders showed an intermingling of protease sequences from the two time-points (Fig. 3 *a*). Interestingly, there was a correlation between the changes in quasispecies and the reduction in DNA sequence diversity after the introduction of therapy.

Quasispecies changes in the *env* gene accompanying the development of genotypic indinavir resistance

The quasispecies changes found in the protease gene during indinavir therapy in those patients where a population bottleneck was observed raised the question of whether these protease gene modifications were also found in other genomic regions, such as the *env* gene. A similar quasispecies analysis to that performed with the protease gene was also done with the C2–V3 *env* gene region. Viral RNA from plasma samples obtained at the beginning of treatment and after 12 weeks were amplified by RT–PCR. Products were cloned and 10–15 clones from each sample were sequenced (Fig. 2 *b*).

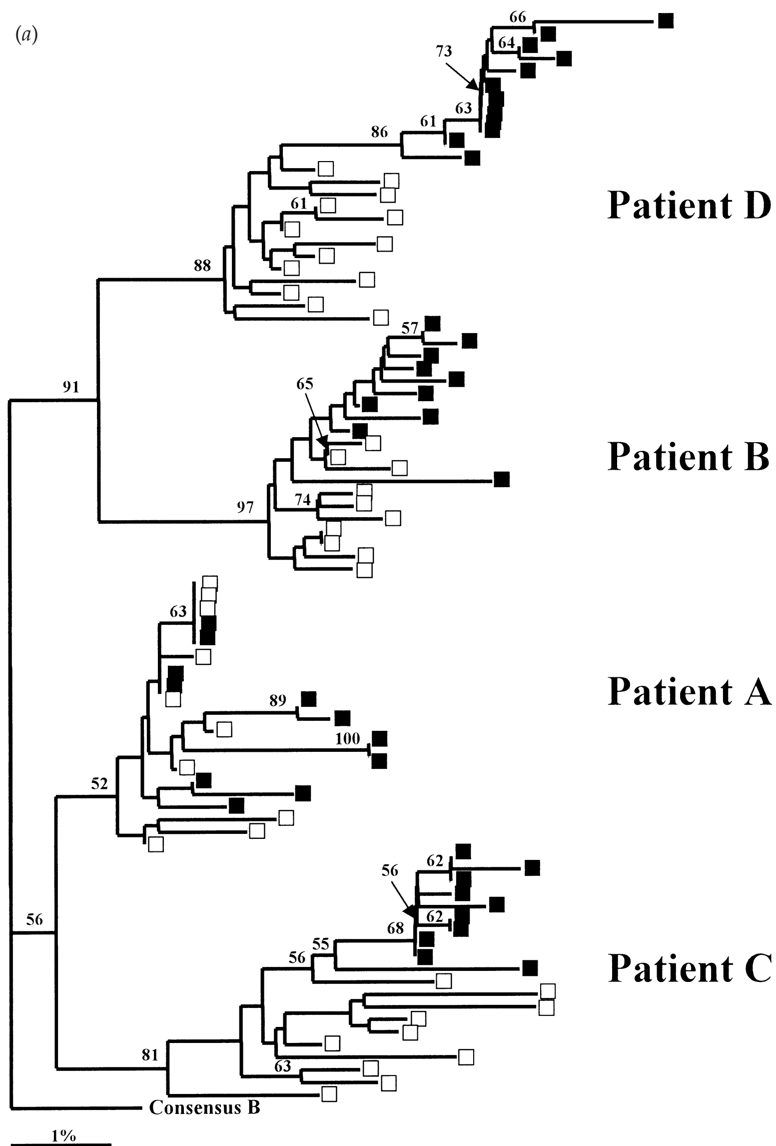
Similar to the results found within the protease gene, the rate of quasispecies evolution (inter-sample sequence distances) (Table 2) in the responder patients (4·9 ± 1·8) was higher than for the non-responders (1·5 ± 0·8) ($P < 0·001$). Likewise, a statistically significant intra-sample DNA sequence distance reduction after therapy was also observed in the transient-responder patients C ($P < 0·001$) and D ($P < 0·001$) (Table 4). This time, a significant intra-sample DNA sequence distance reduction after therapy was also observed within the non-responder patients A ($P = 0·008$) and B ($P < 0·001$). When the synonymous and nonsynonymous nucleotide substitution patterns were computed for the *env* quasispecies (Table 4), significant increases in the ds/dn ratio were observed after 12 weeks of indinavir therapy for patients A, C and D. This increase indicates that there was an absence of selective pressure for amino acid changes within this *env* coding region during this period.

Phylogenetic reconstruction of the *env* sequences showed a similar situation to that found in the protease gene. The two

Table 3. Intra-time-point distances for protease DNA sequences

Intra-time-point comparisons of synonymous substitutions per potential synonymous site (ds) and nonsynonymous substitutions per potential nonsynonymous site (dn) and the ratio of synonymous to nonsynonymous base substitutions (ds/dn). Any sequence that lacked a nonsynonymous substitution (i.e. dn = 0) was not included in the calculation. Values are means ± SD. Probabilities were derived from Wilcoxon's signed rank test. NR, Non-responder; TR, transient responder.

	DNA distance			ds			dn			ds/dn		
Patient	Initial	Final	*P*	Initial	Final	*P*	Initial	Final	*P*	Initial	Final	*P*
A (NR)	1·6 ± 1·4	1·7 ± 0·9	0·835	5·2 ± 5·6	2·6 ± 2·3	0·196	0·5 ± 0·8	1·0 ± 0·7	0·010	5·9 ± 2·1	2·1 ± 1·9	0·012
B (NR)	1·4 ± 0·5	1·4 ± 0·9	0·651	4·2 ± 2·3	2·9 ± 2·5	0·004	0·4 ± 0·5	0·8 ± 0·8	0·014	4·3 ± 2·3	2·9 ± 2·5	0·001
C (TR)	3·0 ± 0·9	1·4 ± 1·1	< 0·001	6·2 ± 3·4	0·8 ± 1·2	< 0·001	2·1 ± 1·1	1·0 ± 1·0	< 0·001	4·1 ± 4·4	1·5 ± 0·7	0·003
D (TR)	6·0 ± 0·9	1·7 ± 0·6	< 0·001	6·6 ± 3·1	0·8 ± 1·1	< 0·001	0·3 ± 0·5	0·6 ± 0·7	0·057	10·1 ± 6·4	1·9 ± 0·7	0·002



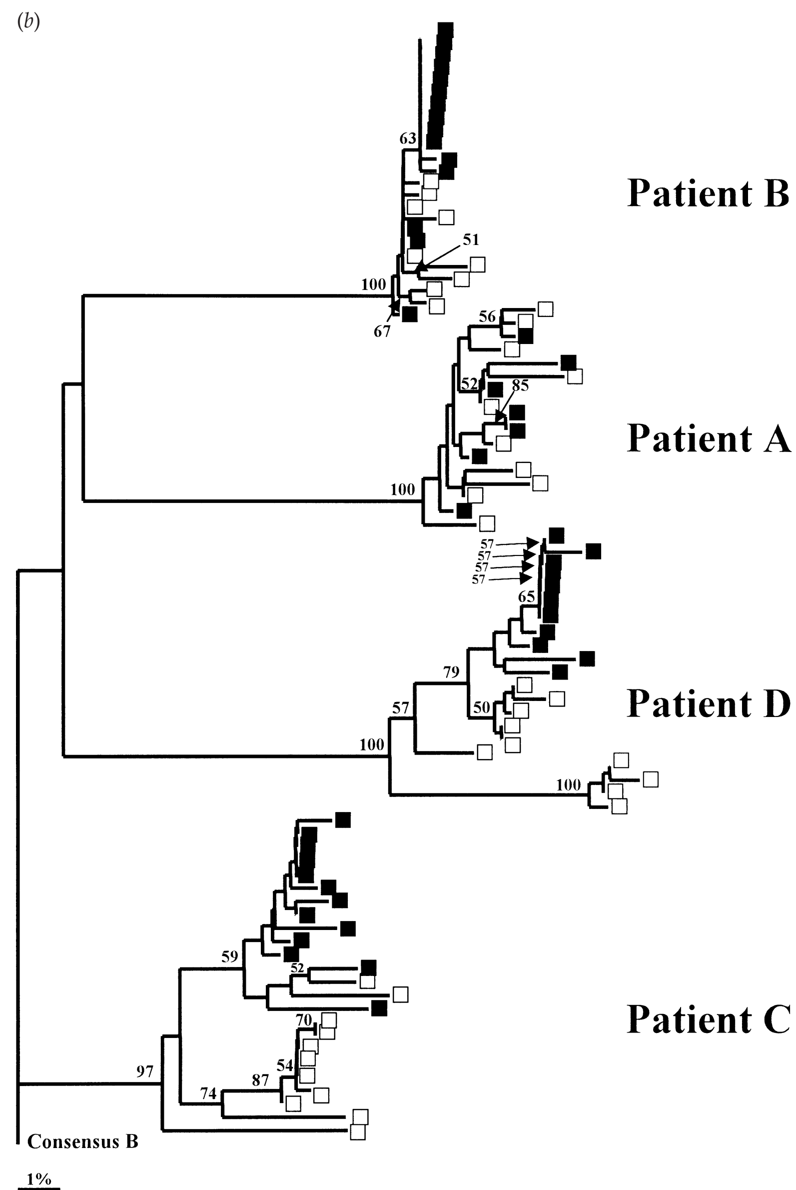


Fig. 3. Neighbour-joining phylogenetic reconstruction of viral nucleotide sequences from the plasma of the four patients analysed in this study. Protease (*a*) and C2–V3 *env* sequences (*b*) from time zero (□) and from the final time-point (12 weeks) (■) are shown. Numbers at branch nodes refer to the number of bootstrap repetitions (of 100) at which the distal sequences grouped together; only those greater than 50% are shown.

Table 4. Intra-time-point distances for *env* sequences

See legend to Table 3 for explanation. NR, Non-responder; TR, transient responder.

Patient	DNA distance			ds			dn			ds/dn		
	Initial	Final	*P*	Initial	Final	*P*	Initial	Final	*P*	Initial	Final	*P*
A (NR)	2·5±0·9	1·5±0·8	0·008	2·6±1·4	2·6±2·3	0·799	2·5±1·2	1·0±0·7	< 0·001	1·2±0·8	3·3±2·8	0·048
B (NR)	1·2±0·6	0·3±0·3	< 0·001	2·8±1·2	0·3±0·6	< 0·001	0·8±0·3	0·2±0·3	< 0·001	4·4±2·4	3·3±1·1	0·042
C (TR)	2·7±2·3	1·8±1·4	< 0·001	6·2±5·5	6·9±5·3	0·853	3·2±2·8	0·8±1·0	< 0·001	1·9±1·0	6·4±3·7	< 0·001
D (TR)	4·3±3·2	1·1±1·0	< 0·001	3·3±2·1	1·5±1·9	< 0·001	4·7±3·5	0·8±0·9	< 0·001	1·3±1·6	4·2±4·1	0·008

transient-responder patients C and D showed a drift in their *env* virus populations after the introduction of therapy (Fig. 3*b*). Interestingly, clusters of eight and four *env* sequences, respectively, present at time zero within the patient C and patient D quasispecies were no longer detected after 12 weeks of therapy (Figs 2*b* and 3*b*). Again, population changes were not found in the non-responder patients A and B. Patient A showed a more heterogeneous virus population, but with intermingling of sequences from the two time-points. Patient B showed a homogeneous population at time zero and at 12 weeks of indinavir therapy (see also Table 4). In order to rule out the possibility that the highly homogeneous quasispecies found in patient B after 12 weeks of therapy may have been derived from some PCR bias or contamination, we re-analysed the *env* sequences from this patient. The sequences obtained from six independent RT–PCR products (Figs 2*b* and 3*b*; Table 4) confirmed the homogeneity of the *env* virus population found in patient B after 12 weeks of indinavir therapy.

Taken together, these results suggest a temporal relationship between the beginning of antiretroviral therapy, a drastic reduction in virus load and a drift in the HIV-1 quasispecies.

Discussion

The present study has characterized virus sequence variation and evolution in four HIV-1-infected patients subjected to indinavir monotherapy. Two of these four patients (patients A and B) showed no decrease in their virus load during the study period (Fig. 1). Two additional patients (C and D) exhibited a rapid decline in their plasma viraemia after the initiation of the therapy, but a virus rebound to time-zero values was observed after 12 weeks. Genetic and phenotypic indinavir resistance was detected in these four patients after 24 weeks of indinavir therapy (Fig. 2*a*; Table 1). As expected, different resistance genotypes were detected in the four patients (Condra *et al*., 1995, 1996). Furthermore, different genotypes were also present within the same patient (Fig. 2*a*), showing that different resistance genotypes coexisted at the beginning of the therapy. Nevertheless, the selected substitution V82A, previously documented to be critical for the development of indinavir resistance (Boden & Markowitz, 1998), was detected in the four patients (Fig. 2*a*). We asked whether the emergence of genetic resistance soon after the beginning of antiretroviral therapy had influenced the genotype of other virus loci not implicated in the rise of resistance to the therapy.

We have shown that the HIV-1 population changes at the *env* locus during the emergence of resistance to indinavir were only observed in those patients (C and D) where the development of resistance occurred with a drastic reduction in virus load (Fig. 1). Therefore, although patients A and B developed genetic and phenotypic resistance to indinavir, the absence of population bottlenecks in these two subjects resulted in the absence of a temporal association between the beginning of antiretroviral therapy and a shift in the virus quasispecies. Interestingly, at time zero, these four patients had no apparent differences in terms of $CD4^+$ T cell count or virus load (Fig. 1), the four subjects being in an advanced stage of HIV-1 disease. Furthermore, the four patients had similar drug levels during the study period (Ruiz *et al*., 1998) and no apparent differences in the occurrence of genetic and phenotypic resistance were observed between the two patient groups (Fig. 2*a*; Table 1). In addition to the changes at the *env* locus during the emergence of resistance to indinavir, we also detected a decrease in the genetic heterogeneity in the two loci analysed, protease and *env*, in the transient-responder patients (C and D). We have previously described a similar result in patients with prolonged suppression of plasma viraemia who, after 24 months of combination therapy, showed a reduction in their PBMC *env* virus population diversity (Martínez *et al*., 1999). Interestingly, in both studies this decrease in *env* genetic diversity was accompanied by an increase in the ds/dn ratio, probably reflecting purifying negative selection expected for populations that are not subjected to significant immunological selection (Lukashov *et al*., 1995; Wolinsky *et al*., 1996; Liu *et al*., 1997).

Changes in the genetic composition of the plasma *env* virus population during the emergence of resistance to a protease inhibitor, similar to that found here for patients C and D, have

recently been documented (Nijhuis *et al*., 1998). The changes in *env* observed in this report were correlated with the amplification of a few drug-resistant viruses. An earlier study, using heteroduplex mobility assays instead of DNA sequence determination, also found changes, although this time transient, at the *env* locus of HIV-1 populations during the emergence of protease-inhibitor resistance (Delwart *et al*., 1998). In both studies, these changes in the populations are explained by the existence of a small effective population size. Assuming a larger virus population size, the observed genetic bottleneck would not be expected because the selection and amplification of a large pre-treatment resistant population would not produce changes in a genetic locus not directly implicated in the emergence of resistance (Leigh-Brown, 1997; Leigh-Brown & Richman, 1997; Nijhuis *et al*., 1998; Delwart *et al*., 1998). However, the former reports failed to analyse the impact of emergence of resistance in the absence of a reduction in virus load. The absence of a reduction in virus load during the emergence of genetic resistance to indinavir, found for patients A and B in the current study, is difficult to explain in the light of the small HIV-1 population size mentioned above. Similar results to those documented here for patients A and B have been reported during AZT treatment (Leigh-Brown & Cleland, 1996; Cleland *et al*., 1996; Sanchez-Palomino *et al*., 1996). In these studies, an impact on the evolution of the *env* gene was not observed during the emergence of resistance to the therapy.

Although many studies have been carried out on HIV-1 evolution, controversy remains as to whether the observed HIV-1 genetic diversification is influenced more by positive selection events (i.e. selection of *env* variants to evade the host immune system: Coffin, 1995; Wolinsky *et al*., 1996) or by genetic drift (i.e. antigenic or cytokine stimulation and amplification of $CD4^+$ T cells: Cheynier *et al*., 1994, 1998; Plikat *et al*., 1997). The data presented here, which show that the population bottleneck that originated during indinavir therapy can select changes randomly in genomic regions not implicated in the emergence of resistance, suggest that virus bottlenecks during intra-patient transmission might be also important in the stochastic intra-patient evolution of HIV-1. Significantly, genetic bottlenecks can contribute to heterogeneity and divergence among populations and to the fixation of mutations independently of their selective value (Sanchez-Palomino *et al*., 1993; Domingo *et al*., 1996; Yuste *et al*., 1999). For instance, a temporal correspondence has been shown between the appearance of virus with lower cytopathogenicity and the emergence of drug resistance during saquinavir monotherapy (Ercoli *et al*., 1997). Virus population bottlenecks might be important in HIV-1 pathogenesis because some differences in virus replication capability in drug-resistant mutants could cause substantial variation in transmissibility and persistence. Estimation of the virus replication capability is important, since it has been postulated that less-fit viruses might give rise to a clinical benefit (Coffin, 1995). Indeed, long-term non-progressing HIV-1-infected patients appear to have less-fit, non-syncytium-inducing viruses than progressors (Blaak *et al*., 1998). However, the high virus loads, close to those found at time zero, detected here for patients C and D, in which an effective population bottleneck was observed, show the ease with which the drug-resistant virus can develop compensatory substitutions to improve its fitness (Nijhuis *et al*., 1997; Martínez-Picado *et al*., 1999). Future investigations are needed to determine the long-term clinical impact of the bottlenecks originating during the treatment of HIV-1-infected patients.

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