Zidovudine treatment results in the selection of human immunodeficiency virus type 1 variants whose genotypes confer increasing levels of drug resistance

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High level resistance to 3'-azido-3'-deoxythymidine (AZT, zidovudine or Retrovir) is conferred by the presence of four or five mutations (Met-41 → Leu; Asp-67 → Asn; Lys-70 → Arg; Thr-215 → Tyr or Phe; Lys-219 → Gln) in the human immunodeficiency virus (HIV) reverse transcriptase. The order of appearance of these five mutations in asymptomatic patients during therapy has been studied. This has enabled us to propose a model for the acquisition of zidovudine resistance mutations during the treatment of high-risk asymptomatic HIV-infected individuals. A consistent acquisition pattern of mutations at codons 41, 70 and 215 was observed in 17 individuals. Complex mixtures of HIV species containing different combinations of single and linked double resistance mutations were present early in zidovudine therapy in isolates from two patients studied in detail. From these mixtures the linked Leu-41/Tyr-215 genotype outgrew all others initially. The development of each new virus population is likely to be mediated primarily by the increase in the level of drug resistance rather than changes in the growth kinetics of the virus. This leads us to conclude that one major driving force in the outgrowth of different mutant viruses is the selective advantage conferred by higher levels of drug resistance.

Introduction

Human immunodeficiency virus (HIV) isolates resistant to many compounds targeted to the virus-encoded enzyme reverse transcriptase (RT) have been identified during drug therapy or by selection in vitro (Fitzgibbon et al., 1992; Larder, 1992; Nunberg et al., 1991; Richman et al., 1991a; St Clair et al., 1991). Variants of HIV highly resistant to zidovudine (AZT, Retrovir) have been isolated from AIDS patients receiving prolonged treatment (Boucher et al., 1990; Land et al., 1990; Larder et al., 1989; Richman et al., 1990; Rooke et al., 1989). High level resistance to zidovudine can be mediated by the acquisition of four specific amino acid substitutions in the RT of HIV-1 (Asp-67 → Asn; Lys-70 → Arg; Thr-215 → Tyr or Phe; Lys-219 → Gln) (Larder & Kemp, 1989). The relative sensitivities of certain combinations of these mutations have been reported (Larder et al., 1991). The appearance of the four amino acid substitutions has been studied in an asymptomatic patient population (Boucher et al., 1992a); this revealed an ordered but complex pattern of mutation acquisition. The substitution of Arg-70 → Lys is commonly detected first during zidovudine treatment. This mutation is only transient, appearing early during drug treatment and then disappearing as the mutation at codon 215 appears. The Lys-70 mutation then reappears, and finally mutations at codons 67 and 219 may develop with continuing treatment. A similar pattern of mutation acquisition was observed in a larger cohort of HIV-infected individuals at various stages of disease (Richman et al., 1991b). More recently a fifth mutation of Met to Leu at codon 41 has been shown to contribute to the development of zidovudine resistance (Kellam et al., 1992). Analysis of an asymptomatic patient population revealed that the mutation at codon 41 was present in 12 of 16 patients after 76 weeks of drug therapy (Kellam et al., 1992).

These studies indicate that complex interactions of mutations in RT are taking place in the virus population, involving gross changes in genotype and phenotype during the course of treatment. The occurrence of rapid sequence changes in the envelope glycoprotein of HIV-1 consisting of regular replacement by a succession of distinct viral populations has been demonstrated (Simmonds et al., 1991). Similar sequence variations have been observed in other HIV gene sequences (Delassus et
al., 1991; Meyerhans et al., 1989; Pedroza Martins et al., 1991; Shen Pang et al., 1991). This has led to the view that HIV pathogenesis involves the rapid evolution of virus strains (Goodenow et al., 1989; Simmonds et al., 1991).

Here we report the detailed genotypic analysis of virus from infected peripheral blood lymphocytes (PBLs) from 17 initially asymptomatic HIV-positive individuals at all five codons associated with zidovudine resistance. Further, we report an extended genetic analysis of virus isolates from two patients from this cohort. A DNA sequence study of the RT-coding region from sequential isolates from these two patients revealed the appearance and turnover of various genotypes during treatment, resulting in the selection of variants with increasing resistance to zidovudine. Construction of a large panel of site-directed mutant viruses was undertaken to provide defined drug sensitivities of the genotypes observed, and to assess the in vitro growth properties of different mutants. The results presented enable us to propose a model for the acquisition of the five mutations that confer resistance to zidovudine.

Methods

Patient cohort. The Amsterdam asymptomatic cohort studied consisted of 18 high-risk (CD4 < 500) HIV-infected individuals of whom 17 were studied (Boucher et al., 1992a). From this cohort two individuals, Patient 199 (P199) and Patient 105 (P105), for whom multiple, sequential, cryopreserved PBL samples were available were chosen for detailed genetic analysis of viral zidovudine resistance.

Detection of Met-41 → Leu mutation by PCR. Selective PCR, used to discriminate wild-type (Met) from mutant (Leu) at codon 41 in co-cultured PBLs, was performed essentially as described previously (Kellam et al., 1992) with the exception that different diagnostic oligonucleotides were used. To identify wild-type codon 41, the primer pair used was BR (5’ GTG GTT GCT CAT CCT CC 3’) with primer SWR (5’ TA CTA GAA ATT TGT ACA GAC A 3’). To identify mutant codon 41, primer BR was paired with either 5MR (5’ TA CTA GAA ATT TGT ACA GAC T 3’) to detect a CTG codon or 5MCR (5’ TA CTA GAA ATT TGT ACA GAC C 3’) to detect a CTG codon 41. The PCR reaction mixture (100 μl) consisted of 25 mM-KCl, 1.5 mM-MgCl₂, 50 mM-Tris-HCl pH 8.3, 0.1 mg/ml BSA, 0.2 mM of each of dATP, dCTP, dGTP and dTTP, 0.25 μM of each primer and 2.5 units Taq polymerase (Cetus). The PCR cycle consisted of 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, 2 min at 50 °C, and 4 min at 72 °C with finishing at 10 min at 72 °C. The second PCR involved primers 5RTA (5’ TTG CAC TTT GAA TGC TCC CAT TAG 3’) and 5RTB (5’ TTA CTA ATT GTG GAA TCC CCC CAT TAG 3’) paired with 3RT (5’ CTT ATC ATC TAT TCC ATC ATC TAG AAA TAG T 3’) and used 1 mM-MgCl₂ in an otherwise unaltered PCR reaction mix. Ten μl of the first PCR reaction was re-amplified under the same conditions except that the extension time at 72 °C was 2 min rather than 4 min. This resulted in a full-length RT sequence with EcoRI and XhoI restriction enzyme sites introduced 5’ and 3’ respectively. The RT sequences were cloned using these sites into the vector pSP73. RT gene-containing clones were sequenced according to the protocol of Sanger & Smith (1984) using Sequenase 2.0. Primers RT9 (5’ AGT ACT GTT ACT GTT TT 3’) and Sp6 (5’ GTA TTA GGG ACT ATA A 3’) were used to sequence base pairs 90 to 255 containing the zidovudine resistance codons 41, 67 and 70. Primers RT11 (5’ TAT GGA GTA GAT TTA GCA G 3’) and RT6 (5’ TTA TGT TAG TGC TTT GG A 3’) were used for sequencing of base pairs 600 to 825 containing the resistance codons 215 and 219.

Cells and virus. The human T lymphoblastoid cell line C8166 (Salahuddin et al., 1983) was used to propagate HIV and for electroporation experiments. These cells were routinely maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, plus antibiotics (RPMI 10). HT4LaCZ-1 cells (Rocancourt et al., 1990) were used to determine the sensitivity of HIV to zidovudine. This cell line was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, plus antibiotics. MT-2 cells (Harada et al., 1985) were co-cultivated directly with patients’ cryopreserved PBLs in RPMI 10 to obtain HIV stocks for sensitivity testing as described (Larder et al., 1989). Patient PBLs were also co-cultivated with donor (HIV seronegative) PBLs and DNA was extracted for PCR and DNA sequence analysis (Larder et al., 1991). To produce site-directed mutant virus stocks, C8166 cells were co-electroporated with a mixture of the RT-deleted proviral clone pHIVARTBstEII and a functional mutant RT gene sequence derived from M13 clones as described (Kellam et al., 1992; St Clair et al., 1991). Cell-free virus supernatants were harvested 12 to 14 days post-transfection and stored at −70 °C.

Construction of mutant viruses. Mutants containing various combinations of the five zidovudine resistance mutations were created by site-directed mutagenesis (Zoller & Smith, 1984) of the previously described M13 RT clones HXB-2D, RTMF, RTMC/F and RTMC (Larder et al., 1991). All mutants were verified by nucleotide sequence analysis (Sanger et al., 1977). M13 replicative form DNA was prepared, and the mutant RT-coding regions were transferred into the HXB-2D genetic background by homologous recombination as described above.

Zidovudine sensitivity assay. Plaque-reduction assays were performed by infection of HT4LaCZ-1 cell monolayers as described (Chesebro & Wehrly, 1988; Larder et al., 1990). The 50% inhibitory concentration (IC₅₀) values were derived from plots of percentage inhibition against zidovudine concentration.

Analysis of virus growth properties. C8166 cells (4 × 10⁸) were infected with 4 × 10⁶ p.f.u./ml of each mutant virus (Table 2). Six infections were set up per mutant. Virus was allowed to adsorb at 37 °C for 1 h. All six infections were then washed, and the cells were pelleted and washed twice with 1 ml of PBS. The cells were then resuspended in 6 ml of RPMI 10, and 1 ml aliquots were plated out in 24-well tissue culture dishes. Samples were taken at time 0 (immediately after plating), 24, 48, 62, 70 and 88 h. At each time point a 1 ml aliquot was removed from each well and the cell-free supernatant was stored at −70 °C. After all samples had been taken the amount of infectious virus produced at each time point was determined by titrating the supernatants on
HT4LacZ-1 monolayers. As controls, the growth properties of the clinical isolate PO26A (Larder et al., 1989) and of wild-type HXB-2D were determined.

Results

Acquisition of the five mutations conferring resistance to zidovudine

Seventeen initially asymptomatic HIV-positive individuals have been studied by selective PCR during the course of zidovudine therapy for the appearance of specific mutations at the RT gene codons 67, 70, 215 and 219 associated with zidovudine resistance (Boucher et al., 1992a). This study was further extended by the analysis of codon 41 in these patients' PBL populations. All co-cultured PBL samples obtained before treatment were wild-type at codons 41, 70 and 215. In addition, for three patients analysed, codons 67 and 219 were also wild-type before treatment (Table 1). A general pattern of resistance mutation acquisition could be observed. In eight of 17 patients (patients 385 to 276, Table 1) the first mutation to appear was at codon 70 either as a mutant population or a mixed population of wild-type and mutant sequences (Boucher et al., 1992a). However, with continued treatment this mutation returned to a wild-type or mixed population. As this occurred the mutation at codon 215 appeared, closely followed by the acquisition of the mutation at codon 41. Both mutations remained in the PBL population once present. In five of 17 patients (patients 105 to 346) a similar pattern was observed with the acquisition of mutations at codons 41 and 215. By 76 to 132 weeks of zidovudine therapy 13 of 17 (76%) patients harboured HIV with mutations at codons 41 and 215. It is likely that for patients 105 to 346 the transient appearance of a mutation at codon 70 occurred between the times of sampling. This was indeed the case for patient 105 who acquired a mutant codon 70 population between 12 and 24 weeks (Fig. 2). For patient 181, a mixed population of wild-type and mutant sequences at codons 70 and 215 arose by 28 weeks and these became mutant at codon 215 alone by 96 weeks. For patient 232 a mutation at codon 215 was observed only after 112 weeks of drug therapy, all other codons remaining wild-type. For patients 56 and 1123 populations with a mutation at codon 70 only were observed.

Detailed analysis of two patients

The temporal appearance of mutations associated with zidovudine resistance in individual patients was indicated from the analysis of 17 asymptomatic HIV-positive individuals. In order to determine more accurately the exact order of appearance of the five resistance mutations, isolates from two patients previously described, Table 1. Longitudinal analysis of RT genes during treatment of 17 HIV-positive individuals with zidovudine (AZT)

<table>
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<tr>
<th>Patient code*</th>
<th>Duration of AZT therapy (weeks)</th>
<th>Reverse transcriptase codon†</th>
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<td>X W W W W</td>
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<tr>
<td>1123</td>
<td>112</td>
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* For patients who progressed to AIDS during treatment, the time AIDS developed is indicated.
† W, wild-type codon; X, mixture of wild-type and mutant codon; M, mutant codon; M*, alternative CTG codon for the mutation at codon 41.

Pt199 and Pt105 (Boucher et al., 1992a), for whom multiple longitudinal samples were available during zidovudine therapy, were analysed by selective PCR.
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Zidovudine Genotype Clonal Frequency Predicted
therapy IC50 (µM) (by PCR analysis) resistance
(weeks) (fold increase)

<table>
<thead>
<tr>
<th>Therapy (weeks)</th>
<th>Zidovudine IC50 (µM)</th>
<th>Genotype (by PCR)</th>
<th>Clonal analysis</th>
<th>Frequency (%)</th>
<th>Predicted resistance (fold increase)</th>
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<tr>
<td>0</td>
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<td>1</td>
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<td>30</td>
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<td>100</td>
<td>64</td>
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<tr>
<td>88</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
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<tr>
<td>109</td>
<td>-</td>
<td>-</td>
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<tr>
<td>132</td>
<td>-</td>
<td>-</td>
<td></td>
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</table>

Fig. 1. Genotype and drug sensitivity analysis of multiple sequential samples from Pt199. Zidovudine sensitivities were assessed in the HeLa CD4+ plaque reduction assay. Mutations associated with zidovudine resistance were monitored by selective PCR. Mutations are shown by shadings of bars: [ ], wild-type detected; [ ], both wild-type and mutant detected; [ ], mutant only detected. The linkage patterns of resistance mutations were determined by sequence analysis of cloned RT coding sequences. The frequency and predicted increase in drug resistance above wild-type is shown for each genotype identified.

(i) Patient 199
A homogeneous population with a mutation at codon 70 was detected by PCR at 12 weeks of therapy (Fig. 1). Subsequently a complex population of mutant codon 70 and mixtures of wild-type and mutants at codons 41 and 215 arose after 36 weeks of therapy. This was followed by the development of mixtures of mutants and wild-type at all three codons by 53 weeks. By 60 weeks of treatment, homogeneous populations were present with mutations at codons 41 and 215, and at codon 70 a mixed population of wild-type and mutant variants was still present. The mutation at codon 70 had disappeared completely after 88 weeks of therapy, and subsequently reappeared as part of a mixed population of mutants and wild-type by 132 weeks.

(ii) Patient 105
The first mutation detected was at codon 70 after 12 weeks of therapy and was present as a mixed population of wild-type and mutant sequences which became completely wild-type at codon 70 as mutations at codons 41 and 215 occurred by 24 weeks (Fig. 2). By 36 weeks a homogeneous population of mutants at codons 41 and 215 had developed. Also at this time the mutation at codon 70 reappeared in a mixed population of mutant and wild-type. This mixture at codon 70 persisted until 136 weeks of treatment when the population became homogeneously mutant. In Pt105 the mutation at codon 67 developed, first as a mixture between 60 and 109 weeks and then subsequently returned to wild-type. The mutation then reappeared as a homogeneous mutant population along with the mutation at codon 219. This resulted in Pt105 having mutations at all five resistance codons at 136 weeks.

It is interesting to note that in both patients the sequence of mutation appearance was very similar but the timing of the appearance of mutations was quite distinct, with Pt105 exhibiting a faster rate of mutation acquisition. At all times of sampling the patients harboured syncytium-inducing isolates of HIV as shown by their ability to replicate in the T cell line MT-2 (Boucher et al., 1992a). Virus stocks prepared were assayed for sensitivity to zidovudine (Fig. 1 and 2). The level of drug resistance increased with continuing drug therapy resulting in the acquisition of a virus with a highly resistant phenotype in Pt105 by 136 weeks.

Clonal sequence analysis of mutation acquisition

PCR analysis revealed the appearance of complex mixtures of wild-type and mutant sequences at various times of drug treatment. From these mixtures changes in the overall genotype of the population arose. Therefore, we wished to determine whether there were linkage
patterns of the five mutations in the changing populations. To address this, DNA sequence analysis was performed on cloned RT sequences from Pt199 at 12, 36, 53 and 132 weeks of drug therapy. The predominant wild-type strain detected at the start of drug therapy was taken as the reference sequence when making sequence comparisons. The frequency of the appearance of each population is shown in Fig. 3 and Fig. 4 for patients 199 and 105 respectively, with sequences grouped with respect to their zidovudine resistance genotype.

(i) Patient 199
At 12 weeks of treatment a mixed population of predominantly wild-type sequences (Group I) of different populations was observed, along with the presence of a population of RT genes containing a mutation at codon 70 (Group II, C199/12/4). By 36 weeks, a complex mixture of mutant genotypes was seen. Ten clones were sequenced and populations consisting of unlinked amino acid substitutions at codons 41 (Group III, 1/10) and 70 (Group II, 3/10) were seen, as well as populations of RT genes with linked mutations at codons 41 and 215 (Group VI, 3/10), or 70 and 215 (Group V, 3/10). By 53 weeks of treatment the population had shifted to contain all linked 41 and 215 mutations (Group VI, 7/7), and this genotype persisted at 132 weeks. Interestingly, at 53 weeks two of seven variants sequenced contained the alternative CTG codon for leucine at amino acid 41 (C199/53/1) rather than the more frequently observed TTG codon. From all the sequence variation observed, amino acid substitutions resulted only at the zidovudine resistance codons and at codon 210 (Leu → Trp).

(ii) Patient 105
At 8 weeks before the start of drug therapy all strains sequenced were wild-type with respect to the known zidovudine resistance codons. Four clones sequenced at this time point revealed the presence of three different wild-type virus genotypes. By 24 weeks of treatment mutations at codons 41 and 215 were observed. In 50% (five) of the clones sequenced the mutations at codons 41 and 215 were linked on the same RT coding sequence, namely (Group VI) C105/24/1 and -2. The remaining clones contained unlinked mutations at codons 41 (Group III, 3/10) or 215 (Group IV, 2/10). With continued drug therapy the appearance of mutations at codons 67 and 70 was observed at 60 weeks. At this time...
Fig. 4. Regions of RT coding sequence for multiple clones from PI105 at -8, 24, 60 and 136 weeks of zidovudine treatment. Nucleotide substitutions differing from the predominant time -8 sequence (shown in full) are indicated. The frequency of a given sequence type is shown on the right and the number of clones (n) sequenced at each time is indicated. The grouping of sequences was based on the identified zidovudine resistance genotype, Group I containing wild-type sequences, Group II containing Arg-70 sequences, Group III containing Leu-41 sequences, Group IV containing Tyr-215 sequences, Group V containing linked Arg-70 and Tyr-215 sequences, Group VI containing linked Leu-41 and Tyr-215 sequences, Group VII containing linked Leu-41, Asn-67, Arg-70 and Tyr-215 sequences, and Group VIII containing sequences with all five zidovudine resistance mutations. Nucleotide substitutions conferring amino acid changes are indicated IS], with zidovudine resistance mutations being shown as D.

a population of linked 41 and 215 mutations (C105/60/1 and -2) remained but the predominant genotype at this time consisted of linked mutations at codons 41, 67, 70 and 215 (Group VII). Interestingly, no linked mutations at codons 41, 67 and 215 or 41, 70 and 215 were seen. By 136 weeks a predominant sequence group containing all five resistance mutations was observed (Group VIII). From all of the sequence variation present, amino acid substitutions occurred only at the zidovudine resistance codons and at codons 44 (Glu -- Asp), 214 (Phe -- Leu), 228 (Leu -- Pro) and 246 (Leu -- Pro). These results with respect to the zidovudine resistance mutations are summarized in Fig. 1 and 2.

Analysis of site-directed mutant viruses

In order to understand why certain mutant virus populations outgrow others in the face of zidovudine therapy, a series of site-directed mutant viruses was constructed with different combinations of the five resistance mutations. Virus recovered was assayed for sensitivity to zidovudine (Table 2). The first resistance mutation detected was Lys-70 to Arg (HIVRTMJ). This had previously been shown to result in an increase in drug resistance of eightfold (Larder et al., 1990). Virus containing only a single amino acid substitution at codon 41 of Met to Leu (HIVRTML) has been shown to exhibit a fourfold increase in zidovudine resistance (Kellam et al., 1992). The double nucleotide substitution resulting in the amino acid change of Thr-215 to Tyr (HIVRTMF) resulted in a 16-fold increase in resistance (Larder et al., 1990).
Sequence analysis of Pt199 and Pt105 RT coding regions showed that these single mutations became linked relatively early in drug therapy to give rise to different double mutants. Site-directed mutant viruses containing these combinations revealed large differences in sensitivity to zidovudine. The combination of mutations at codons 41 and 215 (HIVRTMN) was shown to result in a greater than additive increase in resistance (64-fold) compared with the mutations singly (Kellam et al., 1992). The combination of Arg-70 and Tyr-215 (HIVRTMO) resulted in a threefold decrease in the level of resistance relative to Tyr-215 alone. The combination of Leu-41 and Arg-70 (HIVRTMW) yielded a virus that was only ninefold more resistant to zidovudine than wild-type virus, but still less resistant than Tyr-215 alone. HIVRTMP which has mutations at codons 41, 70 and 215 resulted in a virus with a twofold decrease in resistance relative to HIVRTMN (41 and 215). This particular genotype was not seen in the sequencing of the clinical isolate RT genes. The site-directed mutant virus with mutations at codons 41, 67, 70 and 215 (HIVRTMQ) has been shown to have a highly resistant phenotype (Kellam et al., 1992). Further addition of the mutation at codon 219 to this background (HIVRTMS; 41, 67, 70, 215 and 219) did not result in any appreciable increase in zidovudine resistance. Although both these combinations were seen during the sequence analysis of clinical isolates the significance of the HIVRTMS (41, 67, 70, 215, 219) genotype and the mutation at codon 219 are unknown. The replication characteristics of the site-directed mutant viruses could contribute to the selective outgrowth of the mutant viruses, the growth properties of the site-directed mutant viruses HIVRTML, HIVRTMF, HIVRTMN and HIVRTMS were compared with the parental wild-type virus HXB-2D. Growth analysis was performed between two and four times for each virus with the average virus titre at each time being plotted. Standard error bars are shown for HXB-2D (inset). The same degree of variation was observed with all assays. Differences in growth between HXB-2D and the clinical isolate PO26A are shown (inset).

Replication characteristics of the site-directed mutant viruses

To determine whether the different RT genotypes could contribute to the selective outgrowth of the mutant viruses, the growth properties of the site-directed mutant viruses HIVRTML, HIVRTMF, HIVRTMN and HIVRTMS were compared with the parental wild-type virus HXB-2D. Growth analysis was performed between two and four times for each virus with the average virus titre at each time being plotted. Standard error bars are shown for HXB-2D (inset). The same degree of variation was observed with all assays. Differences in growth between HXB-2D and the clinical isolate PO26A are shown (inset).
significant at 20 h p.i., and peak virus production was reached at about 40 h.

**Discussion**

Information gathered from this study and previous work on zidovudine resistance enables us to propose a model for the pattern of resistance mutation acquisition in high-risk asymptomatic HIV-infected individuals (Fig. 6). Changes in virus populations over time can be attributed to a change in the virus’s ability to replicate in a given environment. This can be achieved by an increase in the replication rate of a virus in the absence of drug therapy, and/or a selective advantage conferred on drug-resistant virus in the presence of the drug. Growth analysis in the absence of zidovudine of site-directed mutant virus containing different combinations of zidovudine resistance mutations revealed no dramatic differences in their replication rates in the T cell line C8166. Gross differences in the ability of HIV-1 strains to replicate could be shown in the assay system used, as demonstrated by differences between the clinical isolate PO26A and HXB2-D. Other cell lines were not studied and could possibly reveal differences in growth rates. Other studies have shown that wild-type virus has a very slight growth advantage over a Tyr-215 (HIVRTMF) mutant in MT-2 cells. In these experiments wild-type virus was able to outgrow and become the dominant virus from an initially mixed population with Tyr-215 (HIVRTMF) after 20 passages in vitro (Boucher et al., 1993). Taken together, these results suggest that the mutant viruses replicate sufficiently similarly in vitro that no mutant would quickly outgrow another. Three groups have reported the gradual return to wild-type of resistant HIV after the cessation of zidovudine therapy (Albert et al., 1992; Boucher et al., 1993; Land et al., 1991). All showed that the change from mutant to wild-type isolates does not occur rapidly and may be dependent on the duration of therapy. This was suggested to indicate that the in vivo replicative capacities of resistant variants are similar to those of sensitive variants (Albert et al., 1992). Therefore, the growth rates conferred by resistance mutations in RT alone are unlikely to contribute dramatically to the change in virus populations during the development of drug resistance.

Each new virus population that becomes dominant does however have a significant increase in resistance to zidovudine, resulting in a selective growth advantage. The first mutation to appear, at codon 70, confers the highest level of resistance (eightfold) of all the mutations due to a single nucleotide substitution (Larder et al., 1990; Kellam et al., 1992). During the initial stages of zidovudine resistance development additional populations of mutants with single amino acid changes co-exist, namely Leu-41 or Tyr-215, the latter conferring the highest level of resistance of all the single amino acid substitutions (16-fold). Different combinations of these mutations can become linked to give double mutants conferring varying levels of drug resistance. Some of these combinations were observed in the clinical samples studied, namely 70/215 and 41/215. The linked phenotype Leu-41/Tyr-215, conferring a greater than 60-fold increase in resistance, outgrows all other mutants initially, and appears to be a major landmark during the development of zidovudine resistance. Of the isolates from two patients studied in detail all became homogeneously mutant at these codons between 36 and 60 weeks of therapy. Of a total of 17 patients studied (including Pt105 and Pt199) 13 had homogeneous populations of linked 41/215 after 76 to 132 weeks of zidovudine treatment. The appearance of homogeneous populations of mutants at the remaining three codons occurred only in Pt105 after 136 weeks. The transition from the double mutant 41/215 to the highly resistant variant 41/67/70/215 could proceed via the intermediates 41/70/215 and/or 41/67/215, although neither of these genotypes was seen during the sequence analysis. It is possible the 41/70/215 and 41/67/215 genotypes occur clinically, as other combinations of mutations that confer lower
resistance levels were observed during the sequence analysis, for example 70/215. The 41/70/215 and 41/67/215 genotypes may be able to replicate well in different cellular reservoirs. In these reservoirs the maximum zidovudine concentration may vary from the level attainable in PBLs (Perno et al., 1992). When these genotypes acquire further mutations and become highly resistant they would then have a selective advantage and outgrow the 41/215 double mutant in the general population. It is important to remember, however, that the patients in this study were a selective subgroup of HIV-1-infected individuals and different patterns of mutations may arise in other patient groups.

DNA sequence analysis suggested that the continued evolution of zidovudine-resistant genotypes involves the turnover of virus populations during the course of treatment. The presence of different virus populations has been studied for a number of HIV genes, rev (Pedroza Martins et al., 1991), nef (Delassus et al., 1991), tat (Meyerhans et al., 1989) and env (Shen Pang et al., 1991; Simmonds et al., 1991), but similar studies have not been performed on RT. Work on other HIV genes has indicated that no two loci evolve at the same rate (Temin, 1990; Linial & Blair, 1982). It is possible that recombination exists in Ptl05 isolates, contributing to zidovudine resistance, for example the combination in HIV RT have been implicated in zidovudine resistance relative to HIVRTMS. It is interesting to note that of 56 RT genes sequenced from the two patients Pt199 and Pt 105 no evidence of a Phe at codon 215 was obtained. The level of drug resistance of other combinations of mutations with Phe-215 is not known, and the relative frequency of Phe-215 compared to Tyr-215 in patient groups is also unknown. However, it is unlikely that highly resistant viruses that contain a mutation at codon 41 will also contain a Phe at codon 215. Other mutations in HIV RT have been implicated in zidovudine resistance although none has been introduced into defined genetic backgrounds to prove their contribution (Gingeras et al., 1991; Japour et al., 1991; Sheehy & Desselberger, 1993). In this study, variation in RT was also observed resulting in amino acid substitutions. However, it is likely that these changes represent strain variation rather than contributing to zidovudine resistance, for example the mutation Phe-214 to Leu in patient 105 was present before zidovudine therapy, and persisted throughout.

In conclusion, we propose a model for the overall acquisition pattern of the five resistance mutations in asymptomatic patients (Fig. 6). Each new mutant population that becomes dominant has a significant increase in resistance to zidovudine resulting in a selective advantage which enables the virus to replicate and outgrow the previous less resistant variant.

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