Prevalence of transmitted nucleoside analogue-resistant HIV-1 strains and pre-existing mutations in *pol* reverse transcriptase and protease region: outcome after treatment in recently infected individuals

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We retrospectively studied 38 Italian recently HIV-1-infected subjects who seroconverted from 1994 to 1997 to investigate: (i) the prevalence of nucleoside reverse transcriptase inhibitors (NRTI)-related mutations at primary infection; (ii) the proportion of naturally occurring mutations in reverse transcriptase (RT) and protease regions of patients naive for non-nucleoside RT inhibitors (NNRTIs) and protease inhibitors (PIs); (iii) the drug-susceptibility to NRTIs and PIs in subjects with NRTI- and/or PI-related mutations; and (iv) the outcome of seroconverters treated with various NRTIs or NRTI/PI regimens. Baseline HIV-1 plasma viraemia and absolute CD4 count at baseline could not be used to distinguish patients with NRTI- and/or PI-related pre-existing mutations from those with wild-type virus (*P*=0.693 and *P*=0.542, respectively). The frequency of zidovudine-related mutations was 21% in the study period. The response to treatment was not significantly different in subjects with or without genotypic zidovudine-related mutations at primary infection (*P*=0.744 for HIV-1 RNA and *P*=0.102 for CD4 cells). Some natural variation (2.6%) was present within regions 98–108 and 179–190 of RT involved in NNRTI resistance. The high natural polymorphism in the protease region present in our patients was similar to that reported by others. In our study some PI-associated substitutions, thought to be compensatory in protease enzymatic function, could confer intermediate to high PI-resistance. As discrepancies between genotypic and phenotypic results may exist in recent seroconverters, our data suggest that the role of transmitted NRTI- and PI-resistant variants remain to be fully elucidated *in vivo*.

Introduction

The risk of acquiring drug resistance at primary infection has become an emerging public health problem following the widespread use of antiretroviral drugs in developed countries. HIV-1-resistant strains may be acquired both sexually [1–4] and vertically [5,6], although the route of transmission seems to be irrelevant [7–10]. Recent reports have indicated a high proportion of transmitted HIV-1-resistant variants in therapy-naive individuals. In particular, strains that are highly resistant to nucleoside RT inhibitors (NRTI) are present in 1% of these subjects as determined by both genotype and phenotype assays, while non-nucleoside RT inhibitors (NNRTI) -resistant viruses may be found in as many as 15–18% of naive patients [11–14]. Protease inhibitor (PI)-resistant variants have been detected in 1–2% of recently infected subjects, although discrepancies are reported between genotypic and phenotypic results in the presence of a low number of PI-related substitutions [11–14]. However, limited studies have addressed the impact of the consequences of transmission of HIV-1 resistant variants. Moreover, few data are available to clarify the complex relationship between genotypic changes occurring in *pol* gene and phenotypic alteration of drug susceptibility of HIV-1 isolates in recently seroconverted HIV-1-infected individuals.

Our study investigated the prevalence of horizontal transmission of NRTI-resistant strains several years

after zidovudine, didanosine and zalcitabine introduction in clinical practice in Italy. Moreover, we retrospectively investigated the effect of NRTI treatment in subjects with transmitted resistant HIV-1 variants. In addition, we also studied the relationship between genotypic and phenotypic results in our case file.

The extensive use of PIs in seroconverters together with previous observations of natural polymorphism in the protease-encoding region among untreated patients, and the occurrence of sexually transmitted PI-related mutations of the protease region [15–18], prompted us to determine the rate of pre-existing mutations of protease region in recently infected subjects. Moreover, although there is limited use of NNRTIs in our country, we analysed the natural polymorphism of RT regions, encompassing both 98–108 and 179–190 residues, that have been reported to be involved in resistance to NNRTIs [19].

Materials and Methods

Seroconverters

We enrolled a consecutive series of HIV-1 seroconverters from January 1994 to March 1997 at the Institute of Infectious and Tropical Diseases of Milan. HIV-1 infection was determined using ELISA antibodies, and seroconversion was confirmed by Western blot. Subjects with a time delay of >8 months between the last negative and first positive antibody testing were excluded from the study. Seroconverters were followed up and treated according to the independent decision of their clinicians. After June 1996 the seroconverters were treated with HAART following the Italian Guidelines for antiretroviral therapy.

Sample collection and preparation

Peripheral blood mononuclear cells (PBMCs) of infected patients were obtained by gradient separation from EDTA–blood samples. PBMCs were recovered, washed three times in RPMI, centrifuged, pelleted and cryopreserved in aliquots of 5–10×10^6 viable cells.

DNA extraction, amplification and sequencing

Cell-associated HIV-1 DNA from patients' PBMCs was extracted with the Elu-Quick isolation Kit (Schleicher & Schuell). DNA was amplified by nested-PCR using *pol* gene-specific primers to analyse all positions known to confer resistance to zidovudine, didanosine, zalcitabine, lamivudine, stavudine and PIs. Each reaction mixture (100 µl) contained 10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 2.5 U of Ampli*Taq* Gold DNA polymerase (Perkin Elmer) and 50 pmol of each primer. Outer primer pair was 1/2 for a region encompassing protease and RT regions; inner primers for RT region were 3/Seq I and Pia 3/4, as described elsewhere [20]. Inner primers for protease region were PRO-F (nucleotide position 2197–2213, 5′TGTAAAACGACGGCCAGTACAACAACTCCCTCTCA3′) and PRO-R (nucleotide position 2628–2612, 5′CAGGAAACAGCTATGACCAATGGCCATTGTTTAAC3′). 1 µg of DNA was amplified during the first PCR run. The reaction was initiated at 94°C for 9 min (one hold); followed by 38 cycles at 94°C for 30 s, 45°C for 50 s and 72°C for 2 min and a final extension cycle at 72°C for 10 min. The second amplification was performed with 5 µl of the first amplification product. The conditions employed were one hold at 94°C for 9 min, followed by two cycles at 95°C for 30 s, 45°C for 50 s and 72°C for 2 min, then 38 cycles at 94°C for 30 s, 55°C for 50 s, 72°C for 2 min, and a final extension cycle at 72°C for 10 min. Unincorporated primers and nucleotides were removed with QIAQuick (Qiagen). PCR products were sequenced directly using dye-labelled M13 primers and ABI sequencing kit reagents (Applied Biosystem) in the presence of 10% glycerol. A DNA automatic sequencer 377 (Applied Biosystems) was used. Sequence reaction started with an initial cycle at 98°C for 1 min 30 s; followed by 15 cycles at 98°C for 15 s, 55°C for 5 min and 70°C for 2 min; and then 15 cycles at 98°C for 15 s and 70°C for 3 s.

Virus isolation and phenotype evaluation

HIV-1 isolates were obtained by cocultivation: 2×10^6 PBMCs taken from patients were cocultured with 2×10^6 PHA-stimulated (1 µg/ml) PBMCs taken from healthy donors. Once a week, fresh 2-day PHA-prestimulated donor PBMCs were added to the cultures, keeping a 1:1 final cell ratio, for 3 weeks in RPMI medium supplemented with 20% fetal calf serum and 10 U/ml of recombinant IL-2 [21]. The culture supernatants were sampled once a week and cryopreserved at –80°C. HIV-1 p24 antigen was assayed in the culture supernatants using a commercial ELISA (NEN, Life Science Products). Virus phenotype was tested in the MT-2 cell line as described [22].

Quantification of HIV-1 RNA and proviral DNA by a reverse-transcription PCR assay and competitive PCR

HIV-1 copy numbers were determined using a quantitative PCR assay (Amplicor HIV Monitor, Roche Molecular Systems) which is based on a single combined reverse transcription and amplification of a conserved region of the *gag* gene, as well as of an internal standard [23].

Lymphocyte subsets and HIV-1 p24 assay

Diagnostic monoclonal antibodies were employed to

Table 1. Prevalence of transmitted zidovudine-associated mutations in 38 recently seroconverted individuals according to the year of seroconversion and susceptibility of HIV-1 isolates to zidovudine in patients harbouring zidovudine-related mutations

			Zidovudine-associated mutations		Zidovudine IC_{50} (μM)*	
Year	*n*	Patient	RT positions	*n*	t_0	t_1†
1994	8	#3‡	41L	1	0.089	0.0001
1995	14	#13‡	70R	4	0.0025	–§
		#15‡	70R		**2.5**	**10**
		#16‡¶	70R		0.0001	–
		#9‡	70K/R; 219Q		–	–
1996	9	#25	70R	2	–	–
		#23	70K/R; 215		0.01	–
1997	7	#35	215Y	1	–	–

*IC_{50} values represent the mean of two replicates. Values in bold are considered phenotypically resistant (>1 μM).
†6th month.
‡Patients retested for RT genotype at a further time point (6th month).
§Not determined.
¶Patient showed revertion of 70R substitution after 6 months.

assess lymphocyte subsets by means of fluorocytometry, using Coulter (Coulter Electronics) or Ortho (Ortho Diagnostic Systems) cytometers.

Compounds

Zidovudine was obtained in powder form from Wellcome Research Laboratories, UK. It was dissolved in sterile PBS and stored at a concentration of 4 mM in aliquots at –20°C until used. Indinavir and ritonavir were obtained in powder form from Merck Research Laboratories and Abbott Laboratories, respectively. They were dissolved in sterile DMSO and stored at a concentration of 1 mM in aliquots at –20°C until used.

Viral titration

Viral titration was carried out in PBMCs, and the viral titre, measured as the 50% tissue-culture infectious dose ($TCID_{50}$)/ml, was calculated by following the method used by Reed & Muench [24].

Drug-susceptibility assay

RT and PI susceptibilities were determined using a fixed amount of infectious virus (1000 $TCID_{50}$/ml). The isolated virus was used to infect 10^6 PHA-P-stimulated PBMCs that were drug-free (control wells) or pre-treated with four concentrations of zidovudine (0.01, 0.1, 1 and 10 μM) or four concentrations of indinavir or ritonavir (0.005, 0.01, 0.05 and 0.1 μM) in duplicate wells. After 7 days, the HIV-1 p24 antigen level was measured in the cell-free supernatant of the cultures. In all experiments, uninfected drug-treated toxicity controls were maintained at the highest concentrations of zidovudine, indinavir or ritonavir. Cell proliferation and viability was assessed by the Trypan blue exclusion method.

Computer-assisted data analysis

The analysis detected modified RT codons associated with phenotypic resistance, and only the PI-related positions were verified both genetically and phenotypically [19]. Data were assembled, manually proofread and edited using the Factura and Sequence Navigator software program (Perkin Elmer) to discriminate the viral quasispecies. Sequences were aligned with the consensus clade B sequence [25], submitted to Genbank with assigned accession numbers AF114267–AF114337.

Statistical analysis

The virological and molecular data were analysed using Fisher's exact test, or a generalization of it for tables greater than 2×2 (Exact test for R×C tables), and standard non-parametric methods (Kruskal–Wallis and Mann–Whitney tests and Spearman rank correlation coefficient). When patients' results were below the detection limit for the HIV-1 RNA assay in plasma, comparisons of the medians in different groups were conducted considering that HIV-1 plasma viraemia was 200 copies/ml. These analyses were performed using StatView version 4.5 (Abacus Concepts). For the drug susceptibility assay, the 50% inhibitory concentration (IC_{50}) for each viral isolate was determined using dose–effect analysis [26] with the Systat computer software program for Macintosh, version 5.1. Comparison of mutation profiles for PIs among naive and experienced subjects was performed using Fisher's exact test (Epi 6.0, CDC). Analysis of the level of phenotypic drug resistance to the different compounds was carried out using the Student's *t*-test (Systat).

Table 2. *In vitro* susceptibility to indinavir and ritonavir in seroconverted patients harbouring HIV-1 variants with or without protease inhibitor mutations

		IC_{50} (μM)*	
Patient	Protease mutations	Indinavir	Ritonavir
#31	L63P, A71T, V77I	0.01	0.01
#11	L63P	0.005	0.005
#36	L10I, L63P	<0.01	0.0071
#38	L63P, I64V	0.033†	0.043†
#29	L10I, L63P	0.020†	0.056†
#23	No mutations	<0.005	<0.005

*IC_{50} values represent the mean of two replicates.
†Phenotypically resistant (>0.01 μM).

Results

Virological characteristics of patients

In the study period we observed 38 recently infected subjects with a documented seroconversion. Patients had a median time lag between negative and positive anti-HIV-1 antibody testing of 2.8 months (range, 1–8). Risk factors for HIV-1 infection were homosexual contact in 17 cases, intravenous drug use in 10 and heterosexual contact in 11. Eight individuals did not receive antiretroviral therapy during the study. All but three patients who seroconverted after June 1996 were treated with HAART, including two NRTIs and one PI. The remaining seroconverters were mainly treated during the course of the infection with NRTI combinations.

All the patients were studied for HIV-1 isolation and phenotype evaluation. Eleven (28.9%) seroconverters gave negative results for virus isolation at baseline. The remainder showed NSI primary isolates with the exception of two SI isolates. Median HIV-1 RNA copies in plasma were 4.44 $\log_{10}$ (range, 3.11–6.17). CD4 cell median count was 550 cells/mm^3 (range, 300–1326). As expected, the levels of plasma viraemia correlated with HIV-1 isolation (P=0.0079). HIV-1 RNA copies were inversely related to CD4 cell counts with borderline statistical significance (ρ=–0.302, P=0.0662). Although 13 seroconverters displayed very high plasma viraemia (HIV-1 RNA >5 $\log_{10}$) at the time of seroconversion, no correlation was observed between the amount of HIV-1 cell-free virions and time from seroconversion (ρ=–0.184; P=0.2623). In addition, no differences in HIV-1 RNA levels and CD4 cell counts at baseline were detected between the eight subjects with transmitted mutations (see below) and the other patients. The median copies of HIV-1 RNA were 4.75 $\log_{10}$, range 3.11–6.17 in the former group and 4.42 $\log_{10}$, range 3.14–6.12 in the latter (P=0.6937). The median CD4 cell counts were 444 cells/mm^3 (range, 344–1326) and 557 cells/mm^3 (range, 300–1193), respectively.

Genotypic and phenotypic resistance analysis of RT region

Neither didanosine- and zalcitabine-related, or lamivudine- and stavudine-related mutations were detected by complete base-paired sequencing of RT region in the case-file. The overall frequency of zidovudine-related mutations was 21% in the 1994–1997 survey period. The analysis performed revealed a prevalence of 12.5% in 1994, seroconverters (1/8) with a strain bearing a M41L substitution and of 28.6% in 1995 seroconverters (4/14) with variants having four leading mutations (of whose one was K70R plus K219Q). In 1996 the prevalence was 22.2% (2/9); in one subject the predominant variant had a K70R change while the other showed a major species with K70R plus T215Y substitutions. The proportion of patients with zidovudine-related mutations in 1997 was 14.3% (1/7) (Table 1). Five out of eight subjects with transmitted mutations were genetically re-tested 6 months later. Only one, harbouring a strain with 70R mutation, showed the reversion of zidovudine mutant genotype as detected by the predominant species of the viral population.

In vitro susceptibility to zidovudine was assessed in five patients bearing isolates with zidovudine-related mutations (Table 2). At baseline, four out of five isolates were zidovudine-sensitive (IC_{50} mean value±SEM, 0.017±0.011 μM). The analysis of zidovudine resistance for patient 15, who exhibited the K70R mutation alone, revealed a persistently high phenotypic profile both at baseline and after 6 months of observation. It is of note that this patient achieved partial control of viral replication during both zidovudine monotherapy and zidovudine/lamivudine association therapy that was started at a later date. HIV-1 RNA copies were 4.71 and 3.04 $\log_{10}$, while CD4 absolute counts were 467 and 453 CD4 cells/mm^3, at the indicated time points. Patient 3, who had an IC_{50} of 0.089 μM at time 0, was re-evaluated 6 months later and showed a maintenance of the IC_{50} in the sensitive range.

The frequency of natural polymorphism involving 98–108 and 179–190 RT coding portions was evaluated in these seroconverters. One subject (2.6%) displayed a K101E change.

Genotypic and phenotypic resistance analysis of protease region

The frequency of spontaneous changes in HIV-1 protease coding region was analysed in 33 seroconverters. It should be noted that only five subjects (15.2%) had no spontaneous mutations in comparison to the reference strain [25]. Sixteen (48.5%), 10 (30.3%) and two (6%) individuals showed one, two,

Table 3. Virological and immunological outcome after 6 months of treatment in seroconverters with HIV-1 strains bearing zidovudine- or protease inhibitor-related mutations and wild-type virus

	HIV-1 RNA (copies/ml)		CD4 cell counts (counts/mm^3)			Time lag†
Patients	t_0*	t_1	t_0	t_1	Antiretroviral therapy	(months)
(a) *Patients treated with two NRTIs after variable time from conversion*						
#3‡	4.71	2.81	509	386	ZDV/3TC	28
#13‡	6.17	5.77	357	520	ZDV/ddI	2
#15§¶	4.71	3.04	467	453	ZDV/3TC	14
#16‡	4.07	2.70	475	621	ZDV/ddI	28
#25‡	4.82	3.95	980	672	ZDV/ddI	1
#2	4.16	3.72	481	478	ZDV/ddI	15
#11	4.41	4.57	737	653	ZDV/ddI	26
#17	3.60	3.86	644	713	ZDV/ddI	4
#20	4.29	3.34	594	401	ZDV/3TC	7
#31	4.46	4.01	486	166	ZDV/ddI	7
(b) *Seroconverters treated with HAART at seroconversion*						
#23‡	5.09	3.46	344	382	ZDV/3TC/SQV	2
#35‡	3.72	BLD	386	761	ZDV/3TC/IDV	3
#21	4.72	2.62	537	842	ZDV/3TC/SQV	3
#28	5.54	BLD	520	1054	ZDV/3TC/SQV	1
#29§	5.22	3.62	612	650	ZDV/3TC/IDV	1
#32	4.46	BLD	415	707	ZDV/3TC/SQV	3
#33	4.46	BLD	509	1023	ZDV/3TC/IDV	2
#34	4.43	BLD	563	1045	ZDV/3TC/IDV	1
#36	5.13	3.95	733	827	ZDV/3TC/IDV	5
#37	6.06	BLD	746	986	ZDV/3TC/IDV	2
#38§	6.12	BLD	517	922	ZDV/3TC/IDV	1

*t_0, baseline for therapy; t_1, 6 months.
†Time elapsed between HIV-1 seroconversion and treatment (months)
‡Subjects with zidovudine-related substitutions who resulted sensitive in *in vitro* drug assays.
§Patients who showed zidovudine or PI drug resistance.
¶Patient with K70R mutation in RT region, resulting in phenotypic resistance to zidovudine.
Abbreviations: ZDV, zidovodine; 3TC, lamivudine; ddI, didanosine; SQV, saquinavir; IDV, indinavir; BLD, below limit of detection of RT-PCR assay.

and three amino acid substitutions at positions related to PI resistance, respectively. The codon substitutions more frequently involved were L63P/S/Q/T/C, I64V/M, V77I, L10V/I, D60E, M36I/L and A71T/V (found in 27, 7, 6, 5, 4, 4 and 3 cases, respectively).

To investigate the biological role of this high rate of spontaneous mutation, we tested two PIs (indinavir and ritonavir; saquinavir was not available from the proprietary company) in five selected patients who exhibited different PI-related multiple patterns of mutations in their HIV-1 DNA sequences (Table 2). Viral isolates were sensitive to indinavir in three out of five subjects (IC_{50} mean value±SEM, 0.008±0.002 μM), whereas they were moderately resistant in the other two cases (IC_{50} higher than 0.01 μM). As for ritonavir, three out of five isolates were sensitive (IC_{50} mean value±SEM, 0.007±0.002 μM) and two were moderately resistant or resistant (IC_{50} mean value±SEM, 0.076±0.012 μM), with statistically significant higher values in the latter cases (Student's *t*-test P=0.028). However, the limited number of patients did not allow us to correlate patterns of phenotypic resistance with genotypic changes. The only patient who exhibited a full phenotypic resistance to ritonavir (patient 38) showed a genotypic profile with both mutations at codons 63 and 64. Patient 29, who showed 63P and 10I substitutions, was partially resistant to PIs. To rule out the possibility that these results could be an artifact due to the differences between viral isolates and the species amplified by PCR on proviral DNA, we sequenced HIV-1 isolates that were used to perform the susceptibility assays from patients 38 and 29. The analysis of the sequences of both patients' isolates revealed a complete base pair identity with their respective direct sequence counterparts.

Outcome of mutation-bearing seroconverters after treatment

Thirty out of the 38 seroconverters received various antiretroviral regimens that were established at seroconversion or later. Three seroconverters received zidovudine alone in 1994 and 1995, 13 patients were treated with two nucleoside analogues (10 subjects in 1994–1995 and three in 1996) while 11 patients were subjected to highly active antiretroviral therapy (HAART, two NRTIs and one PI) at seroconversion.

Table 3 shows the HIV-1 RNA and CD4 cell counts at baseline and after 6 months of treatment in 10 subjects receiving similar combinations of NRTIs and 11 patients treated at seroconversion with HAART. All therapeutic regimens included zidovudine. No statistically significant differences were present in subjects, with or without transmitted zidovudine-related substitutions who received two NRTIs as starting regimens when virological and immunological profiles were considered. The median counts of plasma HIV-1 RNA and CD4 lymphocytes were 4.71 $\log_{10}$ (range, 4.07–6.17) or 4.29 $\log_{10}$ (range, 3.60–4.46), and 475 (range, 357–980) or 594 cells/mm^3 (range, 481–737) at baseline, respectively. After 6 months of therapy the median values of plasma viraemia and CD4 cells were 3.04 $\log_{10}$ (range, 2.70–5.77) versus 3.86 $\log_{10}$ (range, 3.34–4.57), and 520 (range, 386–677) versus 478 cells/mm^3 (range, 166–713), respectively (Table 3a). Patients receiving three drugs showed a rapid decrease in HIV-1 plasma viraemia, with values below the detection limit of the assay in seven out of 11 cases after 6 months. At the same time, an increase in the CD4 cell count was observed (Table 3b). One subject with zidovudine-related mutations (patient 23) showed a modest reduction in plasma viraemia (1.63 $\log_{10}$) and a 10% increase in CD4 cells. However, this viral isolate was sensitive to zidovudine and had neither PI-related mutations, or evidence of phenotypic resistance to indinavir and ritonavir. The other subject (patient 35) showing zidovudine-associated changes reached undetectable levels of HIV-1 plasma viraemia with an increase of 375 CD4 cells/mm^3. Among seroconverters treated with HAART at seroconversion, three additional subjects (patients 11, 29 and 36) did not achieve undetectable levels of HIV-1 RNA. Their CD4 cell counts increased by 57%, 6% and 13%, respectively. It should be noted that patient 29 was found to be phenotypically less susceptible to indinavir. In contrast, patient 38 whose isolate was resistant to PIs, had a marked reduction in plasma viraemia and a CD4 cell count increase of 405 cells/mm^3.

Discussion

Transmission of zidovudine-resistant HIV-1 variants was first reported in 1993 [1]. Various studies have demonstrated that 215F/Y RT mutations rose in the USA from 1.4% in 1991 to 7.5% in 1992 and 10.4% in 1993–1994 [7].

Early European data reported discrepancies that might depend upon: (i) molecular methods used to detect HIV-1 genetic mutations of *pol* gene; while direct sequencing procedures distinguish both major and minor HIV-1 species, the selective PCR, albeit very sensitive, and the Line Probe Assay (LiPA), do not provide information regarding the proportion of different wild-type or mutated viral species [9,10]; (ii) the viral compartments studied; cell-associated HIV-1 that was amplified in several surveys may not be equivalent to cell-free virions that sustain HIV-1 replication and spread of infection to target cells [2–4, 9,10]; (iii) time of survey and the relative restricted use of NRTIs in some countries in comparison to others [2–10, 27].

In our survey of newly infected individuals, neither HIV-1 plasma viraemia or CD4 absolute count at baseline distinguished between patients with NRTI- and/or PI-related pre-existing mutations, and those with wild-type virus. The response to treatment was not influenced by the presence of zidovudine-related mutations in the absence of phenotypically resistant isolates. Our data suggest that about 20% of patients infected after 1993 in the Milan area had zidovudine-resistance related mutations. No difference was observed among patients who belong to specific risk groups. Similar to observations made in the USA, an increasing prevalence may be expected as a reflection of the widespread use of this compound that requires a relatively long time to develop a degree of resistance because of the large number of mutations needed to overcome the 'genetic barrier'. Notably, the leading zidovudine-related K70R was the most represented mutation in our group of patients. This mutation was observed in six out of eight subjects with a predominance in 1995, when regimens were frequently represented by zidovudine alone or in association with another nucleoside analogue. The absence of didanosine- and zalcitabine-related mutations may be due to the limited use of these drugs in comparison with zidovudine, as well as to the low frequency of genetic changes conferred by these compounds. The lack of a rapid lamivudine-induced M184V substitution may depend upon both the time of sampling and the fast reversion of this mutation as detected *in vitro* [28] and in plasma samples (unpublished data). Our data on zidovudine-related resistance showed that four out of five seroconverters with a transmitted genotypic variant exhibited a fully sensitive phenotypic profile, suggesting that isolates with this virological pattern might respond to future antiretroviral treatment including zidovudine. The clinical outcome of our patients confirmed this hypothesis.

Although several studies aimed to define a correlation between genotypic and phenotypic resistance and virological failure [29–32], no final conclusions can be drawn at present. Recent data by Wegner *et al.* [11] indicated that the separate analysis of genotype and phenotype in antiretroviral therapy-naive subjects may provide conflicting results. This author found that when the genotypic data suggest a 'possible resistance' (8% of the cases), the phenotypic results indicate the

absence of PI resistance. One interpretation of this finding may be that these subjects showing genotypic mutations have a low number of changes in the protease region. In addition, in these surveys the phenotypic resistance to NRTIs was not fully predicted by genotypic results. The overall frequency of NNRTI polymorphisms detected in our case-file was 2.6%, suggesting some spontaneous variation in RT regions involved in NNRTI resistance. The effect of regimens containing one NNRTI cannot be predicted on the basis of present data.

Several studies indicate that patients subjected to combination therapy including PIs showed a genotypic profile with pre-existing mutations at the baseline [15–17]. These substitutions involve positions that are specifically selected by the use of PIs. Although a large number of amino acid substitutions were evident in our study, the naturally occurring amino acid changes were entirely represented by secondary mutations [33] that may play a role in conferring PI-resistance. Our results confirm the occurrence of a large polymorphism in the protease-coding region in recently infected individuals and suggest that naturally occurring substitutions may be involved in *in vitro* drug-resistance [34]. With regards to patients investigated for phenotypic susceptibility to PIs, three out of five patients with one to three PI-related secondary mutations showed a sensitive phenotype. Surprisingly, two of these patients presented a moderate to high resistance phenotype that could not be explained by the possible differences among sequences of HIV-1 proviral DNA in comparison to isolates obtained by co-cultivation. Nevertheless, in one case we observed a partial response to triple-drug therapy including a PI, while in the other a marked control of viral replication was seen after 6 months of therapy with HAART. In addition, some of the pre-existing mutations correspond to changes that are compensatory in protease enzymatic function and their role in drug-naive subjects remains to be established [35]. The administration of potent combination regimens might have controlled the growth of the viral strains less susceptible to PIs that were detected before therapy. Moreover, our analysis (direct sequencing) did not allow us to quantify the phenotypically resistant variants. An additional consideration, as suggested by Wainberg *et al.* [7], is that resistant mutants may be disadvantaged in terms of growth compared to wild-type virus *in vivo* in recently infected patients not exposed to antiretrovirals.

Even though anecdotal, our data suggest that further information is needed in order to define the correlation between the presence of secondary mutations of protease *pol* region and its consequences on phenotypic resistance and therapeutic benefits. We must take into consideration that HIV-1 resistant variants may be preferentially transmitted compared to other strains [7,18] and that little is known about the selective fitness of these variants.

After considering that a certain proportion of transmitted drug-associated mutations is emerging over time, we believe that periodic surveys are required to define the magnitude of transmitted antiretroviral drug-resistance and to help clinicians in making therapeutic decisions in HIV-1 acute infection.

Acknowledgements

We thank Mrs Elizabeth L. Kaplan for editorial assistance. All the patients gave their informed consent to participate in the study. The study was conducted in accordance with Italian Ministry of Health Guidelines.

This work was supported by grants from Istituto Superiore di Sanità, X Progetto AIDS # 30.A.0.43 and 40.A.0.72 to MM.

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Received 16 August 1999; accepted 29 November 1999