Resistance of HIV-1 to antiretroviral agents in blood and seminal plasma: implications for transmission

Joseph J. Eron, Pietro L. Vernazza, David M. Johnston, Françoise Seillier-Moiseiwitsch, Timothy M. Alcorn, Susan A. Fiscus and Myron S. Cohen

Objectives: To evaluate blood and genital secretions from HIV-infected men for HIV-1 resistant to antiretroviral agents.

Design: A longitudinal study of 11 men with HIV infection and persistent detectable HIV RNA levels in blood and semen on antiretroviral therapy.

Methods: HIV-1 from the blood and seminal plasma, obtained before the initiation of a new therapeutic regimen and on therapy, were evaluated by population-based sequencing of reverse transcriptase (RT) and protease RNA for the development of resistance to antiretroviral therapy. The genetic relatedness of sequences over time was compared.

Results: RT genotypic resistance markers were present in seminal plasma at baseline in three out of six individuals with previous RT inhibitor experience. Eight out of 10 men, from whom the viral sequence was available on new therapy, demonstrated the evolution of new resistance mutations in the blood or seminal plasma, or both. The evolution of resistance mutations in blood and semen were frequently discordant, although over time similar patterns were seen. In two individuals, protease inhibitor resistance mutations evolved in the blood but not in the major variant in seminal plasma. Comparisons of the viral sequences between blood and seminal plasma from six men revealed two patterns. Three men showed a clustering of sequences from blood and semen. Three had sequences that appeared to evolve separately in the two compartments.

Conclusions: HIV-1 variants with genotypic resistance markers are present in the male genital tract and evolve over time on incompletely suppressive antiretroviral therapy. The absence of genotypic changes consistent with protease inhibitor resistance in the semen, despite their presence in blood plasma, suggests the possibility of limited penetration of these agents into the male genital tract. Sexual transmission of resistant variants may have a negative impact on treatment outcome in newly infected individuals and on the spread of the diseases within a population. Therapeutic strategies that fully suppress HIV-1 in the genital tract should be a public health priority.

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From the Departments of Medicine, Microbiology and Immunology, and Biostatistics at the University of North Carolina at Chapel Hill, North Carolina, 27599-7030, USA, Laboratory Corporation of America, Center for Molecular and Pathology, 1912 Alexander Dr., RTP, NC 27709, USA; and Institut fur Klinische Mikrobiologie und Immunologie, Frohbergstrasse 3, CH-9000 St. Gallen, Switzerland.

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Requests for reprints to: Joseph J. Eron Jr. MD, Associate Professor of Medicine, CB 7030, Division of Infectious Diseases, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7030, USA.

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Introduction

The treatment of HIV-1-infected individuals with combination therapy has resulted in a significant suppression of HIV replication and improvement in clinical outcomes in a substantial proportion of patients [1,2]. Antiretroviral therapy is likely to influence the sexual transmission of HIV-1 because the inoculum is also almost certainly an important determinant of sexual transmission, as it appears to be for parenteral and vertical transmission [3–5]. Therefore effective antiretroviral therapy may also decrease the spread of HIV-1 within a population.

Semen is the primary vehicle for the sexual transmission of HIV-1 from men to their sexual partners [6]. HIV-1 RNA can be quantified in the seminal plasma (SP) of most men who are infected with HIV [7–10]. The level of HIV RNA in SP correlates with the recovery of HIV-1 in culture from seminal cells, although amplification-based, RNA quantification techniques are more sensitive than culture [8,10]. The level of HIV-1 RNA in SP is correlated with the plasma RNA level and inversely correlated with the CD4 cell count [8]. In addition, factors such as genital tract inflammation associated with sexually transmitted diseases (STD) substantially raise HIV-1 RNA levels in semen [11].

Antiretroviral therapy lowers HIV-1 RNA levels in semen and decreases the ability to recover infectious virus [9,12–14]. The magnitude of decline in the HIV RNA level in SP with therapy is usually similar to the effect of treatment on the blood viral burden. Failure of treatment to suppress HIV RNA levels completely in the blood is common [15], resulting in the selection of HIV-1 variants in the blood resistant to the agents used in therapy [16,17]. Not all men on antiretroviral therapy have complete suppression of HIV-1 replication in their genital tract, and they may shed resistant HIV-1. In this study, HIV-1 protease and reverse transcriptase (RT) sequences in the blood and SP from men with detectable RNA in both compartments, despite antiretroviral therapy, were analysed for mutations known to be associated with RT and protease inhibitor resistance. A phylogenetic analysis of HIV-1 RNA sequences was performed.

Methods

HIV-1-positive men at the University of North Carolina (USA) or the Kantonsspital, St Gallen (Switzerland), who had detectable HIV RNA levels in the blood and semen at the start of a new antiretroviral therapy and had subsequent positive values on therapy were studied. All the men gave written informed consent in accordance with Institutional Review Board policy at the respective institutions. HIV RNA levels from these individuals have been reported elsewhere [8,12,14]. Men gave semen samples with or without blood samples at the time of therapy initiation and then at one or more times while on therapy. Antiretroviral therapy was administered according to specific treatment trials or the patient's physician.

Sample collection and processing

Semen samples were collected and processed as previously described [7,10]. Blood samples were collected in acid citrate dextrose (ACD) tubes, processed within 4 h of drawing, and plasma was frozen at −70°C until analysis.

HIV-1 RNA measurements

HIV-1 RNA in the SP was measured as previously described, using the quantitative NASBA method (Organon-Teknika, Boxtel, the Netherlands), which has a lower limit of detection of 1000 RNA copies per ml [10,18]. In blood plasma, HIV-1 RNA was quantified using the NASBA assay or the Amplicor™ HIV-1 Monitor™ Test (Roche Diagnostic Systems, Inc., Branchburg, NJ, USA) with a lower limit of detection of 1000 copies per ml for NASBA and 400 copies per ml of the Roche assay.

HIV-1 RNA sequence analysis

HIV RNA was extracted from blood plasma or SP using the Qiagen Viral RNA Extraction Kit. Viral RNA was reverse transcribed using AMV RT, and a portion of the pol genomic region, including all of the protease gene and the first 242 codons of the RT region, was amplified by a single polymerase chain reaction (PCR) step with the Affymetrix HIV PRT T3 and T7 primers (Affymetrix, Santa Clara, CA, USA). Labelled cRNA generated using either T7 or T3 polymerases and fluoroscein-dUTP was hybridized to Affymetrix HIV PRT GeneChip Probe Arrays according to the manufacturer's instructions. Hybridized probe arrays were scanned on a Hewlett Packard Gene Array Scanner confocol laser microscope, and sequence data was generated using the Affymetrix GeneChip 2.0 software with the Rules algorithm for base-calling. The amino acid sequence was deduced from the nucleotide sequence. The consensus amino acid sequence of RT and protease used for comparison was from the Los Alamos database. Coding changes from consensus associated with HIV resistance to RT and protease inhibitors were taken from the published literature (reviewed in Reference [19]). ABI sequencing (Applied Biosystems, Foster City, CA, USA) was used to confirm possible coding changes identified by the Affymetrix technique that had not previously been described. Sequences from blood and SP were aligned using a multiple sequence alignment algorithm implemented in the Clustal W software [20]. After the multiple alignment, sequences were visually compared

to ensure appropriate alignment [21]. Phylogenetic analysis was performed using several reconstruction methods featured in Clustal W and Phylip software [22], including maximum likelihood [23], maximum parsimony and the distance method with neighbor-joining [24]. Three separate distance metrics were considered [25–27]. Bootstrap analyses were performed on each phylogenetic tree [28].

Results

Subject description

Eleven subjects, chronically infected with HIV-1, were identified with detectable HIV-1 RNA in the blood and SP before and while receiving a new antiretroviral regimen. These individuals represent 25% of the subjects (n = 44) who began new antiretroviral therapy from whom blood and semen samples were collected over time [14]. Five subjects were naive to antiretroviral treatment. Six subjects had received previous RT inhibitor therapy. All subjects were naive to protease inhibitors. Six subjects began protease inhibitor-containing regimens. Median follow-up was 24 weeks (range 8–58 weeks). Six subjects had sequence available from blood and semen; five had sequence from SP samples only. Immunological and virological parameters of the 11 subjects at baseline are listed in Table 1; as are the maximum changes from baseline observed on therapy in blood and seminal HIV RNA level and in CD4 cell count. The range of HIV RNA levels in blood plasma and SP on treatment were 3.2–6.7 and 3.3–7.7 $\log_{10}$ copies per ml, respectively. In six out of 11 subjects baseline HIV RNA in SP was higher than in the blood plasma. There were no consistent differences in these individuals in CD4 cell counts, symptomatic HIV disease or previous antiretroviral drug therapy compared with the other five subjects. Five subjects had baseline SP RNA of greater than 6.5 $\log_{10}$ copies per ml, whereas only one individual had blood HIV RNA greater than this level.

HIV-1 resistance mutations

Sequence was obtained from 63 out of 66 samples available at the time of this analysis. The three samples (all from semen) that failed to yield amplified product for the sequencing reaction had HIV RNA levels of 3.4, 4.7 and 5.0 $\log_{10}$ copies per ml. Only three of the 63 sequences were obtained from samples with less than 5000 (3.7 $\log_{10}$) copies per ml. Individuals who evolved mutations associated with resistance while on therapy are included in Table 2, which lists the amino acid positions at which changes were seen. Two subjects, one treated with zidovudine/saquinavir and the other with indinavir, had no changes noted at baseline or on therapy and are not included in Table 2. These individuals had minimal sustained changes in HIV RNA levels in the blood on treatment (< 0.5 $\log_{10}$). Subject U7 is also excluded from Table 2 because sequence information after baseline was unavailable as a result of the limited sample volume.

At baseline only one therapy-naive individual (DW) had a change from consensus noted at an RT codon previously described to be associated with resistance [19]. A K65R substitution consistent with zalcitabine (ddC) resistance [29] was present in the blood but not the semen (Table 2). Three out of six subjects, who were treatment-experienced with RT inhibitors, had a variable number of coding changes consistent with known resistance mutations at baseline (D10 and E4 in Table 2 and U7). Subject D10, with extensive previous nucleoside analog treatment, had mutations consistent with zidovudine (ZDV), ddC and lamivudine (3TC) resistance in both blood and SP [30–38]. Subject U7 had RT mutations in virus from SP consistent with resistance to ZDV at RT codons 41 and 215 [31,34] . Resistance mutations to ZDV and nevirapine (NVP) were noted in the semen from subject E4 (Table 2), who had received these agents in the past [17,39]. Two additional subjects (E8 and SR) exhibited ZDV-resistance mutations [30–32] in the semen within 8 weeks on new therapy. These mutations may have been present as a minority variant at baseline.

Variation from consensus was seen at baseline in semen and blood at a small number of protease codons associated with protease inhibitor resistance in these protease inhibitor-naive individuals (Table 2) [16,40–43]. These polymorphisms also occur, however, in the absence of protease inhibitor therapy [44,45].

In eight subjects, resistance mutations emerged on their new treatment regimen (Table 2). In four treatment-naive individuals who received ZDV or ZDV plus saquinavir, ZDV resistance mutations developed on treatment. In one subject (SHU) the K70R mutation, which arises early on ZDV therapy and is associated with loss of antiretroviral effect [30,46], emerged as the

Table 1. Baseline immunological and virological parameters

Immunological and virological parameters	Median	Mean	Range
Baseline CD4 cells/μl (median)			
Baseline blood plasma HIV-1 RNA ($\log_{10}$ copies/ml)			
Baseline seminal plasma HIV-1 RNA ($\log_{10}$ copies/ml)			
Maximum change in CD4 cell count on study (cells/μl)			
Maximum change in blood plasma RNA on study ($\log_{10}$ copies/ml)			
Maximum change seminal plasma RNA on study (log copies/ml)			

Table 2. Changes from amino acid consensus sequence at reverse transcriptase and protease codons associated with resistance to RT and protease inhibitors in study subjects who had resistance mutations emerge on therapy

		Protease							Reverse transcriptase											
SHU	Treatment naive: began ZDV/SQV																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Blood	R†	L	–	V	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Semen	–	–	V	V	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 12	Blood	R	–	P	V	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Semen	–	–	V	V	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 32	Blood	R	–	P	V	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–
	Semen	–	–	V	V	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TM	Treatment naive: began ZDV/SQV																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Blood	–	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Semen	–	*	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 12	Blood	–	*	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Semen	–	*	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 16	Blood	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Semen	–	*	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 24	Blood	sample unavailable for sequencing							–	–	–	–	R	–	–	–	–	–	–	–
	Semen	–	*	P	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–
Week 41	Blood	M	*	P	–	–	–	M	–	–	–	–	R	–	–	–	–	–	–	–
	Semen	–	*	P	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–
Week 48	Blood	–	–	P	–	–	–	M	–	–	–	–	R	–	–	–	–	–	–	–
	Semen	–	–	P	–	–	–	–	–	–	–	–	R	–	–	–	–	–	–	–
DW	Treatment naive: began ZDV																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Blood	–	–	*	–	–	–	–	–	R	–	–	–	–	–	–	–	–	–	–
	Semen	–	–	P	–	*	–	–	–	–	–	–	–	–	–	–	–	*	–	–
Week 4	Blood	sample unavailable for sequencing																		
	Semen	*	–	P	–	*	–	–	–	E	–	–	–	–	–	–	–	–	–	–
Week 12	Blood	*	–	*	–	*	–	–	–	R	–	–	–	–	–	–	–	–	–	–
	Semen	–	–	*	–	*	–	–	–	R	–	–	–	–	–	–	–	–	–	–
Week 40	Blood	–	–	P	–	–	–	–	–	E	–	–	–	–	–	–	–	–	–	–
	Semen	*	–	*	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 56	Blood	*	–	*	–	*	–	–	–	R	–	–	R	–	–	–	–	–	Y	–
	Semen	–	*	*	–	*	–	–	–	E	–	–	–	–	–	–	–	–	Y	–
JM	Treatment naive: began ZDV																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Blood	–	–	S	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Semen	–	–	S	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 8	Blood	–	–	S	–	*	–	–	–	–	–	–	R	–	–	–	–	–	–	–
	Semen	sample failed to amplify for sequence																		
Week 13	Blood	–	–	S	–	*	–	–	–	–	–	–	R	–	–	–	–	–	–	–
	Semen	sample failed to amplify for sequence																		
Week 25	Blood	–		S	–	–	–	–	L	–	–		R	–	–	–	–	–	–	–

Table 2. *Continued*

		Protease							Reverse transcriptase											
Week 29	Semen	–	–	S	–	*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 49	Blood	–	–	S	–	I	–	–	L	–	–	–	–	–	–	–	–	–	W	Y	–
	Semen	–	–	P	–	*	–	–	L	–	–	–	–	–	–	–	–	–	–	Y	–
Week 58	Blood	–	–	S	–	I	–	–	L	–	–	–	–	–	–	–	–	–	W	Y	–
	Semen	–	–	S	–	I	–	–	L	R	–	–	–	–	–	–	–	–	W	Y	–
D10	ZDV, 3TC, ddC, d4T experienced: began RTV																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Blood	–	*	P	–	–	–	–	L	–	–	D	–	–	V	–	–	W	Y	–
	Semen	–	–	P	–	*	–	–	*	–	–	D	–	–	V	–	–	G	Y	–
Week 8	Blood	–	*	P	–	–	A	–	L	–	–	D	–	A	V	–	–	*	Y	–
	Semen	–	*	P	–	–	–	–	L	–	N	D	–	–	V	–	–	G	Y	–
E-4	ZDV, NVP experienced: began ZDV/3TC																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Semen	–	–	P	–	–	–	–	L	–	N	–	–	–	–	L	A	W	Y	*
Week 22	Semen	–	–	P	–	–	–	–	L	–	N	–	–	–	–	L	A	W	Y	N
Week 26	Semen	–	–	P	–	–	–	–	L	–	N	–	–	–	–	L	A	W	Y	*
E-8	ZDV experienced: began ZDV/3TC/IDV																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Semen	–	*	P	–	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 3	Semen	–	*	P	–	I	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 8	Semen	–	*	P	–	–	–	–	L	–	–	–	–	–	V	–	–	W	Y	–
Week 24	Semen	–	*	P	–	*	–	–	L	–	–	–	–	–	V	–	–	W	F	–
SR	ZDV experienced: ddC added, changed to 3TC week 12																			
Codon		20	36	63	71	77	82	90	41	65	67	69	70	75	184	188	190	210	215	219
Consensus		K	M	L	A	V	V	L	M	K	D	T	K	V	M	Y	G	L	T	K
Week 0	Semen	–	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Week 4	Semen	–	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–
Week 12	Semen	–	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–
Week 13	Semen	–	–	P	–	–	–	–	–	–	–	–	–	–	–	–	–	–	Y	–

Amino acid changes from consensus are listed using single amino acid nomenclature. Previous therapies and antiretroviral therapy initiated at week 0 are listed for each subject.
*Sequence position at which a predominant a.a could not be determined. This result may be due either to an inability of the system to make a base determination as a result of nucleotide variation not accounted for by the GeneChip assay or to the presence of a 50–50 mixture of bases encoding for the amino acid at that position. ZDV, zidovudine; 3TC, lamivudine; ddC, zalcitabine; ddI, didanosine; RTV, ritonavir; IDV, indinavir; SQV, saquinavir; NVP, nevirapine. Polymorphisms at protease codons 10, 36, 63, 71 and 77 have been shown to occur in HIV from individuals who have not received protease inhibitor therapy [44,45]. Amino acid substitutions at these codons have also been associated with protease resistance. Examples of polymorphisms in these subjects that have also been associated with resistance include: L63P [16], A71V [40] and V77I [41]. †The K20R mutation has been associated with indinavir and ritonavir resistance, however this substitution has been observed in HIV from other protease inhibitor-naive subjects (C. Pilcher, personal communication).

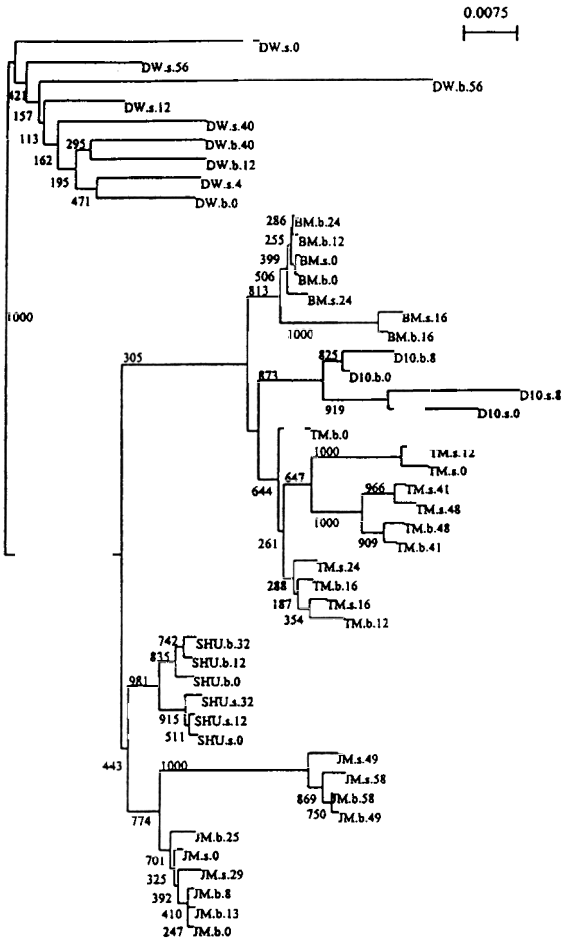

Fig. 1. Sequence from a portion of the pol genomic region including the entire protease gene and 0–242 codons of the reverse transcriptase, from blood and seminal plasma HIV RNA of six subjects were analysed. Sequences were aligned for maximum homology using a progressive multiple sequence alignment method. Phylogenetic analysis was performed using the neighbor-joining method, using all sequences available from individuals who had both blood and semen samples amplified. Horizontal branch lengths are proportional to the estimated divergence (sequence differences) along each branch. Numbers at branch nodes refer to the number of bootstrap repetitions (of 1000) at which the distal sequences group together. Each terminal horizontal branch represents a majority variant from the blood or semen at a given time for a given subject using the following nomenclature: subject.source.week (e.g. JM.s.49: JM is the subject, s is the source compartment [s, semen and b, blood] and 49 is the time on therapy in weeks). Phylogenetic trees constructed using maximum likelihood and maximum parsimony were similar. Phylogenetic analyses including one with a reference strain from the sequencing laboratory and one with NL43 (a laboratory isolate) were undertaken. In each analysis the control variant was distinct from each of the subject groups (data not shown).

predominant sequence in the blood but not in SP. In the other three subjects, ZDV resistance mutations appeared in the blood and semen, but the time at which they appeared, and the number of mutations varied between compartments (Table 2). In one subject (TM), a ZDV resistance mutation became predominant in the semen before its appearance in the blood. 3TC resistance also evolved in the semen in one subject (E8). In two subjects [D10, TM], mutations consistent with protease inhibitor resistance evolved in the blood but not in SP (Table 2). One, the V82A mutation, emerged rapidly on ritonavir therapy (subject D10) [40]. A L90M mutation emerged after 40 weeks on saquinavir therapy (subject TM) [47].

Correlation of HIV-1 RNA sequence in blood and semen

Deduced amino acid sequences from blood and semen samples, obtained at the same times from individual subjects, were 100% concordant in only one blood/semen pair (BM, week 0). All other blood/semen pairs from that subject and from all other subjects had multiple amino acid sequence differences. These differences included protease gene polymorphisms at codons 36, 63, 71 and 77.

The genetic relatedness of nucleotide sequences from all subjects who had blood and semen sequences available for interpretation is shown in Fig. 1. Sequence groups from each individual were distinct from those of all other subjects in each of the analyses performed. Nucleotide sequences from the blood and semen of the same subject, obtained at the same time, were never identical and were frequently separated by substantial genetic distance (Fig. 1).

In two subjects naive to antiretroviral therapy (JM, TM) (Fig. 1), the nucleotide sequence seemed to evolve over time, with sequences from later times in the blood and semen having greater genetic distance from earlier specimens, potentially because of the emergence of resistant variants. In two subjects (SHU, D10), temporal genetic variation in the blood and semen compartments appeared separate between the two compartments (Fig. 1). In subject TM, blood and semen sequences also appeared to be distinct from each other at later times (Fig. 1). These three subjects had differential emergence of resistance mutations in the blood compared with semen (Table 2).

Discussion

For most HIV-1-infected men, potent antiretroviral effects in the systemic compartment appear to result in similar effects in the genital tract [13,14]. These potent effects do not occur in all HIV-1-infected men who are treated. In the cohort of men presented here, resistance to antiretroviral agents, as evidenced by genotypic changes, was easily documented in both the blood and

SP. Subjects naive to antiretroviral therapy were unlikely to have mutations consistent with RT inhibitor resistance, although variations or polymorphisms in the protease sequence [44,45] were seen in the blood and semen at baseline. Subjects who had received previous nucleoside or non-nucleoside RT inhibitor therapy commonly had evidence of resistance in both compartments. Mutations consistent with resistance to ZDV, 3TC, saquinavir and ritonavir emerged in the blood in subjects during treatment, and mutations consistent with resistance to ZDV and 3TC emerged in the semen. The rate and pattern of the emergence of resistance in the two compartments was frequently different. The sequencing technique used in this study provides information on the majority HIV sequence present in the sample. Minority variants may go undetected and the frequency of resistance mutations may thus be underestimated [48].

Individuals in this cohort are a distinct subset of a larger group of subjects starting new antiretroviral therapy [13]. The majority of men with detectable HIV-1 RNA in the semen before the initiation of new therapy had HIV-1 RNA levels in SP fall below quantifiable limits on therapy (19/30 or 63%), including all men who had HIV-1 RNA levels fall below quantifiable levels in the blood. In contrast, subjects who had a poor response to therapy, as measured by changes in HIV RNA concentration in the blood plasma, also had very poor responses in semen plasma RNA. In the subset of men presented here, variable responses to therapy in the blood and seminal RNA were observed (Table 1). No formal measures of adherence were performed in these subjects. Intolerance or poor adherence to therapy may, however, be responsible for the poor antiretroviral responses in this group. More than half had HIV RNA levels in the semen at baseline that were greater than levels in the blood, a finding we have not observed in larger cohorts [8]. In addition, we noted five men, who had seminal HIV RNA values greater than 3×10^6 copies per ml before new therapy, substantially higher than median levels in either the larger group from which these subjects were drawn [14] or in other reported cohorts [8,9,13]. In the study by Coombs *et al.* [9] only two out of 117 men had HIV RNA levels in the semen greater than 3×10^6 copies per ml. Men with very high viral loads in their semen may be at particular risk for the selection of resistant variants in the male genital tract and for persistent shedding of HIV on therapy.

The current study further adds to previous studies demonstrating that HIV-1 in the male genital tract is in a biologically separate compartment, in which the virus is at least partly produced locally and may be under different selective pressures than virus in the systemic compartment [7,11,49]. A phylogenetic analysis of RT and protease sequences demonstrates that the majority variant in SP is almost always distinct from that of the majority variant in blood. Frequently, there is substantial genetic distance between variants obtained at the same time from the two compartments (Fig. 1), as has been seen in small cross-sectional studies [50,51]. In addition, genetic sequences appear to evolve along separate paths in the systemic and genital tract compartments in certain individuals (subjects SHU, D10 and TM (later time points)). These subjects were noted to have differences between blood and semen in the resistance mutations occurring on therapy (Table 2). Differential selective pressures for resistance development in the two compartments may be what is driving the genetic divergence. Differences in antiretroviral drug concentrations between the systemic compartment and the male genital tract may play a role in the differential patterns of resistance emergence. ZDV appears to penetrate the male genital tract well [52], and ZDV resistance mutations as the predominant species in SP can precede, coincide with or follow the appearance of ZDV resistance mutations in the blood (Table 2). Two subjects who developed protease inhibitor resistance mutations in the blood plasma did not develop these mutations in the majority variant in semen. Byrn *et al.* [50] reported a similar finding at one time point in a subject on a protease inhibitor. Whereas the protease inhibitor indinavir reduces the concentration of HIV in the semen [13,53], the degree of protein binding of most protease inhibitors may limit their concentration in genital secretions and selective pressure for resistance development. An increased understanding of the pharmacokinetics and pharmacodynamics of antiretroviral agents in the genital tract is needed.

The incomplete suppression of HIV-1 in the genital tract allows for the evolution of resistant variants in this compartment. The transmission of variants resistant to ZDV, nevirapine and now protease inhibitors has been documented [54–56]. Antiretroviral agents that penetrate the genital tract poorly may allow ongoing replication in this compartment even in the face of apparent effectiveness in the systemic compartment. A subset of men appear to be hypersecretors of HIV RNA in SP, which may be a marker of increased infectiousness [11]. These men may be particularly prone to incomplete suppression of HIV replication in the genital tract and therefore at greater risk of shedding resistant HIV-1. Our observations may have substantial consequences for newly infected individuals and for public health. Although all HIV-infected individuals, regardless of treatment, should be considered potentially infectious, multiple lines of evidence suggest that a lower HIV inoculum results in reduced transmission rates [3–5,57,58]. The reduction of systemic HIV burden greatly benefits individual patients [1,2]. Suppression of HIV in the male genital tract is likely to have public health benefits. Complete suppression of HIV in the

semen may, however, be necessary to prevent the evolution of resistant variants in this compartment and therefore should be a goal of antiretroviral therapy.

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