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High Viral Load in Semen of Human Immunodeficiency Virus Type 1-Infected Men at All Stages of Disease and Its Reduction by Therapy with Protease and Nonnucleoside Reverse Transcriptase Inhibitors

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Seminal viral load is likely to be directly related to the sexual transmissibility of human immunodeficiency virus type 1 (HIV-1). However, it is not clear whether the level of HIV-1 in semen varies with the stage of infection and whether antiretroviral therapy reduces seminal viral load. A nucleic acid sequence-based amplification (NASBA) technique was used to quantify HIV-1 RNA as an indicator of infectious viral load in semen and blood plasma of homosexual men with different stages and durations of HIV-1 infection. The median viral load in a cross section of 34 men was 11,000 HIV-1 RNA copies/ml (range, <400 to 1.3×10^7 copies/ml) in whole semen and 5,238 HIV-1 RNA copies/ml (range, <400 to 2.8×10^5 copies/ml) in seminal plasma, which is 10- to 1,000-fold higher than previous estimates. Viral loads in whole semen and seminal plasma were strongly correlated with blood plasma viral load ($P < 0.001$) but not with blood $CD4^+$ T-cell count ($P = 0.420$). Longitudinal analysis of eight subjects who progressed to AIDS showed that seminal viral load increased in most cases, with viral load consistently higher in blood plasma than in semen. Viral loads in semen and blood plasma decreased markedly in six other patients following initiation of potent combination therapy with a protease inhibitor (indinavir) and a nonnucleoside reverse transcriptase inhibitor (DMP-266). These findings have important implications for the biology of sexual transmission of HIV-1 and its potential reduction by antiretroviral therapy.

Human immunodeficiency virus type 1 (HIV-1) infection in adults is transmitted predominantly by sexual routes. The efficiency of sexual transmission of HIV-1 may depend primarily on the level of infectious virus RNA in semen. By using PCR and virus culture, it has been estimated that each milliliter of semen contains only 10 to 100 copies of HIV-1 RNA (6, 15, 16). Moreover, a lower concentration of HIV-1 has been noted in seminal plasma than in cells, leading to the suggestion that the cellular fraction of semen is the major source of transmitted virus (15, 16). Against this suggestion, however, is the observation that infection of monkeys by vaginal inoculation is 10,000-fold more efficient with cell-free simian immunodeficiency virus (SIV) than with cell-associated SIV (20).

A related and still unclear aspect of HIV-1 infection is whether the level of virus in semen varies at different stages of disease, which could be directly associated with the sexual transmissibility of the virus. Although the quantity of HIV-1 RNA in blood predicts the rate of $CD4^+$ T-cell decline and progression to AIDS (5, 8, 11, 17–19, 25), it is not known whether the viral load in semen correlates with that in blood. There is also very little information available regarding whether antiretroviral therapy can reduce seminal viral load, which could significantly decrease the transmissibility of the virus. It is possible that antiviral drugs do not penetrate efficiently into the male reproductive tract, thus allowing HIV-1 replication to continue at this site of infection.

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In this study we measured viral load using a highly sensitive nucleic acid sequence-based amplification (NASBA) assay in semen and seminal plasma of HIV-1-infected individuals at different stages of disease in cross-sectional and longitudinal studies, and after the initiation of potent antiretroviral therapy. Our data show the presence of substantially higher viral RNA levels in whole semen and seminal fluid at all stages of HIV-1 disease than previously recognized. Furthermore, antiretroviral therapy with a protease inhibitor and a nonnucleoside reverse transcriptase (RT) inhibitor was found to reduce viral load in semen markedly. These results indicate that the transmissibility of HIV-1 is likely to be high throughout the natural course of infection but greatly reduced after therapy with new potent antiretroviral drugs.

Study population and measurement of viral load. For measurement of viral load in the cross-sectional study, paired semen and blood samples were obtained from 34 HIV-1-infected and 10 seronegative homosexual men who were enrolled in the Pittsburgh portion of the Multicenter AIDS Cohort Study, a natural history study of HIV-1 infection in homosexual men (12). Antiretroviral therapies differed among the infected subjects at the time of sampling, i.e., 8 were receiving monotherapy or combination therapy with a nucleoside analog RT inhibitor, 6 were receiving a protease inhibitor alone or in combination with an RT inhibitor, and 13 were not receiving any antiretroviral therapy. Treatment history was not available for the remaining seven subjects. For measurement of viral load in the longitudinal study, paired semen and blood plasma samples were obtained from eight Multicenter AIDS Cohort

Study HIV-1 seroconverters who developed clinical AIDS. Among these eight subjects, five were not receiving antiretroviral therapy during the study period and three were treated either with RT inhibitor or ampligen (1) monotherapy or combination therapy with an RT inhibitor and ampligen. Paired semen and blood plasma samples were also collected from six subjects enrolled in a randomized blinded trial comparing 800 mg of indinavir three times daily (Merck) (7, 26) alone with a combination of 800 mg of indinavir three times daily and 200 mg of DMP-266 (Dupont) once daily (31). Entry criteria for the clinical trial were a $CD4^+$ T-cell count between 100 and 500 cells/mm^3 and plasma HIV RNA levels of at least 20,000 copies/ml.

Semen and heparinized blood plasma samples were processed within 4 to 6 h of collection and frozen at −80°C until testing. Seminal plasma was prepared by centrifugation of whole semen at 1,500 × *g* for 20 min at 4°C. Levels of HIV-1 RNA in whole semen, seminal plasma, or blood plasma were quantitated by the NASBA assay (NASBA HIV-1 RNA QT; Organon Teknika, Durham, N.C.). The NASBA assay was performed on 100 μl of freeze-thawed samples according to the protocol supplied by the manufacturer. Performance characteristics of the assay have been described elsewhere (13, 28, 29). Interassay variation of the assay was 0.13 to 0.23 $\log_{10}$ copies/ml (24, 28). All samples were blind-coded for testing. Spearman correlation coefficient analysis was used for comparisons of viral load determinations and $CD4^+$ T-cell numbers, which were measured in whole blood by staining with fluorescent dye-conjugated monoclonal antibodies specific for CD4 (Becton Dickinson, Mountain View, Calif.) as previously described (3).

High viral loads in semen and seminal plasma and their relation to blood viral load and T-cell counts. Previous attempts to quantify HIV-1 levels in semen and seminal plasma have relied on virus culture and PCR (9, 14, 19, 23, 30). With either of these techniques, only low levels (10 to 100 copies/ml) of HIV-1 have been detected in seminal plasma (6, 15, 16). We attempted to quantitate HIV-1 RNA levels in seminal plasma by an internally controlled, reverse-transcription PCR (RT-PCR) assay (4) and found that seminal plasma inhibited the RT-PCR, as reported by Dyer et al. (2). In a direct comparison of viral load in 14 seminal plasma samples measured by the NASBA assay and the RT-PCR assay, higher levels of HIV-1 RNA were detected by the NASBA assay than by the RT-PCR test in all samples (a median of 3,500 copies/ml and a range of <400 to 140,000 copies/ml by NASBA, compared to a median of 19 copies/ml and a range of <5 to 7,210 copies/ml by RT-PCR). We therefore used the NASBA assay to quantify HIV-1 RNA levels in samples of whole semen, seminal plasma, and blood plasma (13, 29). The linear dynamic range of the NASBA assay is 4×10^2 to 1×10^7 HIV-1 RNA copies per reaction. In initial experiments, we determined that whole semen or seminal plasma did not interfere with HIV-1 RNA quantification by the NASBA assay (data not shown).

Viral load was measured by the NASBA assay in semen and blood plasma samples collected at the same study visit from 34 randomly selected HIV-1-infected homosexual men with a median duration of infection of more than 98 months (range, 30 to more than 144 months). Ten HIV-1-seronegative healthy homosexual men served as controls. $CD4^+$ T-cell counts among the 34 HIV-1-infected men ranged from 37 to 819 cells/μl (median, 338 cells/μl). Table 1 shows that the median viral load was 11,000 copies/ml in whole semen (range, <400 to 1.3×10^7 HIV-1 RNA copies per ml) and 5,238 copies/ml in seminal fluid (range, <400 to 2.8×10^5 HIV-1 RNA copies/ml). HIV-1 RNA was not detected in any of the semen or blood samples from the 10 HIV-1-seronegative controls (data not shown). Repeat testing of aliquots of the same semen samples indicated that the average assay variability was less than twofold (data not shown), which is comparable to that reported by others (13, 24). The median viral load in blood plasma from the 34 individuals was 33,000 copies/ml and the range was <400 to 1.8×10^6 copies/ml, which correlated strongly with the viral loads in whole semen ($r = 0.67$; $P = <0.001$) and seminal fluid ($r = 0.62$; $P = <0.001$). The HIV-1 RNA levels in blood correlated only weakly with $CD4^+$ T-cell counts ($r = -0.27$; $P < 0.01$), as we have previously reported (18). Viral loads in whole semen and in seminal fluid were not significantly correlated with the blood $CD4^+$ T-cell count ($r = 0.16$; $P = 0.42$). We do not have information on the number of mononuclear cells in semen from most of the subjects, because we used cryopreserved whole semen for this study. However, in seven semen samples from Table 1 for which we have data on seminal mononuclear cells, there was no correlation between the number of seminal mononuclear cells and the level of viral RNA in whole semen. Furthermore, there was no clear relationship between seminal viral load and the antiviral treatment regimens that were being administered to a portion of these 34 subjects at the time of this study (data not shown). These cross-sectional results indicate that whole semen and seminal

TABLE 1. Quantitation of HIV-1 RNA in semen and blood by NASBA

Subject no.	No. of $CD4^+$ cells per μl	No. of HIV RNA copies/ml[a] in:		
		Whole semen	Seminal plasma	Blood plasma
1	819	<400	<400	<400
2	779	<400	<400	72,000
3	743	12,000	<400	27,000
4	698	37,000	26,000	19,000
5	694	<400	<400	11,000
6	497	5,900	1,500	96,000
7	494	27,000	16,000	31,000
8	479	6,200	1,500	190,000
9	468	14,000	6,400	33,000
10	456	130,000	40,000	33,000
11	449	900	<400	2,000
12	436	750,000	NA	100,000
13	413	<400	<400	1,000
14	410	490,000	140,000	250,000
15	383	<400	<400	16,000
16	353	14,000	4,300	69,000
17	349	17,000	8,000	46,000
18	326	<400	<400	9,900
19	277	32,000	8,400	NA
20	265	29,000	5,000	160,000
21	220	7,100	1,500	18,000
22	216	10,000	11,000	120,000
23	213	<400	<400	5,600
24	203	1,000	1,450	25,000
25	191	<400	800	1,000
26	187	<400	<400	11,000
27	133	450	550	150,000
28	102	1,700,000	280,000	150,000
29	101	18,000	5,400	120,000
30	72	<400	NA	1,500
31	66	410,000	NA	1,800,000
32	33	13,000	2,800	1,100,000
33	9	12,000	NA	75,000
34	7	13,000,000	NA	76,000

[a] NA, not available.

TABLE 2. Quantitation of HIV-1 RNA by NASBA in whole semen and blood plasma longitudinally obtained from subjects who progressed to AIDS

Subject no.	Time postseroconversion (mo)[a]	No. of $CD4^+$ cells per μl	No. of HIV-1 RNA copies/ml of:	
			Semen	Plasma
117	3	451	<400	3,600
	23	184	<400	NA[b]
	42	301	<400	5,600
	54	101	<400	<400
118	11	782	10,500	190,000
	15	484	16,800	110,000
	22	168	23,400	790,000
119	2	597	<400	650,000
	14	419	<400	250,000
	20	269	5,000	190,000
120	2	1,157	<400	45,000
	14	479	<400	690,000
	26	200	7,600	240,000
	32	152	16,000	94,000
	39	16	790,000	300,000
121	14	1,133	13,666	13,000
	26	540	38,571	52,000
	44	274	177,777	100,000
	50	229	1,291,291	100,000
122	−3	514	<400	<400
	14	518	1,746,988	NA
	32	317	1,139,240	96,000
	38	298	430,434	41,000
123	7	575	4,750	19,000
	33	471	<400	16,000
	45	439	<400	29,000
	57	381	22,000	49,000
124	3	326	18,400	600,000
	20	NA	1,550	220,000
	47	387	13,430	48,000
	71	415	4,199	100,000
	83	347	7,105	130,000
	101	143	5,454	240,000

[a] Patients 117 through 124 developed AIDS at 46, 26, 33, 32, 68, 62, 85, and 124 months postseroconversion, respectively.
[b] NA, not available.

plasma contain high HIV-1 RNA levels at all levels of $CD4^+$ T cell number.

Relation of seminal viral load to the stages of HIV-1 infection. Longitudinal studies have clearly shown that blood plasma viral load increases with the decline in $CD4^+$ T-cell numbers and progression to AIDS (8, 17, 18). To determine whether seminal viral load changes with disease progression, we measured viral loads in whole semen samples collected longitudinally from eight individuals who progressed to AIDS 26 to 124 months after seroconversion (Table 2). In five of these eight progressors (subjects 118 to 121 and 123), viral loads in semen increased as much as 2,000-fold between the first postseroconversion sample and later time points as the disease progressed with decline in $CD4^+$ T-cell counts. Two progressors (subjects 122 and 124) had moderate to high seminal viral loads throughout the follow-up period. One progressor (subject 117) did not have detectable virus in any semen sample. In agreement with prior reports (8, 17, 18), blood plasma viral loads in seven of the eight progressors either increased with disease progression or were maintained at moderate to high levels throughout the follow-up period. The one progressor (subject 117) who had an undetectable semen viral load also had a low blood plasma viral load. There was no association between the antiretroviral therapy (RT inhibitors or ampligen or a combination of both) that some of these subjects received during the course of infection and seminal or blood viral load (data not shown). Seminal plasma was not available for testing from these longitudinal, cryopreserved specimens due to low volumes of sample. These data suggest that the levels of HIV-1 RNA in semen are relatively low in the early phases of infection in many individuals and increase with progression of disease.

Decrease in seminal viral load after antiretroviral therapy. Treatment of HIV-1-infected subjects with new, highly potent antiretroviral therapy, such as protease inhibitors, has been shown to lower the viral RNA load in blood plasma markedly (7, 21). To determine whether such antiretroviral treatment has any effect on seminal viral load, we next examined changes in viral loads in semen and blood from six patients enrolled in a clinical trial comparing monotherapy with indinavir, a potent HIV-1 protease inhibitor (26, 27), with indinavir in combination with DMP-266, a nonnucleoside RT inhibitor (31). Semen and blood samples were obtained before and after initiating antiretroviral therapy. Subject 54 received indinavir monotherapy, and subjects 51, 52, and 53 received combination therapy with indinavir and DMP-266. Subjects 55 and 56 are still in the blinded phase of the clinical trial, and thus their assignment to either the monotherapy or the combined therapy is currently unknown to us. Figure 1 shows that seminal viral loads in all six subjects decreased markedly after 4 weeks of therapy (reductions, >4- to 150-fold). In the three subjects for whom semen samples were available within 2 weeks of the initiation of therapy, 5- to 10-fold decreases in seminal viral load were apparent. These prompt reductions in seminal viral load paralleled the profound decreases observed in blood plasma viral load, which ranged from more than 100- to 650-fold (Fig. 1). The levels of virus in semen and blood remained undetectable (<400 copies per ml) for up to 28 weeks of therapy. The magnitude of the decrease in viral load was greater in blood plasma than in semen, but this differential response may be attributable to the higher initial viral load in blood plasma rather than to a lesser antiretroviral effect in semen. These results indicate that indinavir alone or in combination with DMP-266 can markedly reduce viral load in semen.

Discussion. In this study we have detected high levels of HIV-1 RNA in both whole semen (median, 11,000 copies/ml) and seminal plasma (median, 5,238 copies/ml) by using the NASBA assay. This seminal viral load is 10- to 1,000-fold higher than previous estimates that were based on RT-PCR and virus culture (6, 15, 16). The fact that virus culture had given lower viral load estimates is not surprising, since culture techniques are substantially less sensitive than nucleic acid-based quantification methods, such as RT-PCR and NASBA. Our data also suggest that the lower values previously determined by RT-PCR could be due to a substance(s) in semen that inhibits the RT-PCR. This inhibitor is removed by the RNA extraction method used in the NASBA procedure and therefore does not interfere with the NASBA assay.

It is unlikely that we overestimated the viral load in semen, since HIV-1 RNA quantification was done in comparison with internal HIV-1 RNA standards that are calibrated against virus particles counted by electron microscopy. In addition, the am-

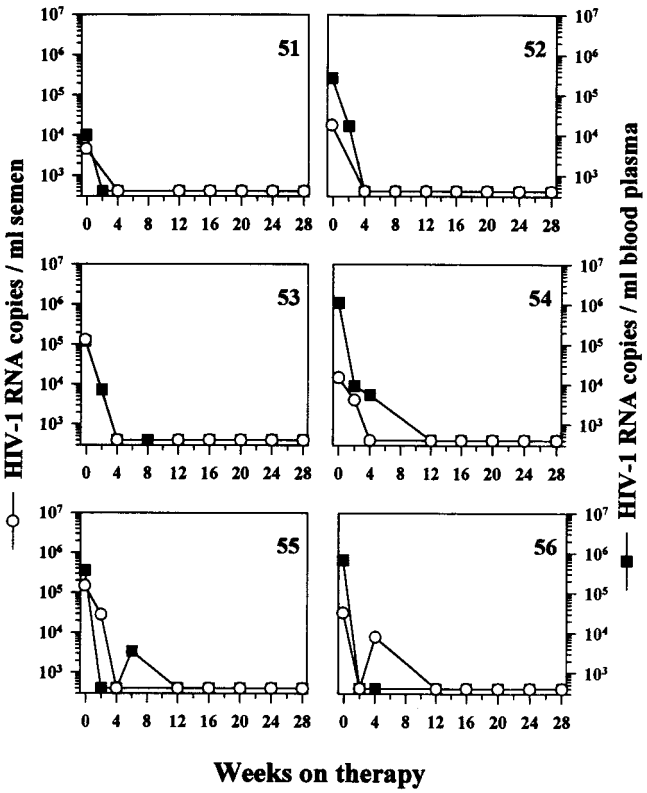

FIG. 1. Effects of antiretroviral therapy on the viral loads in semen and blood plasma. Whole semen and blood plasma were collected at the same clinic visit before, and at the indicated times after, initiation of therapy with indinavir alone or indinavir plus DMP-266 and were tested by the NASBA assay.

plification signals produced by the internal HIV-1 RNA standards were not different when added to whole semen, seminal plasma, or blood plasma, indicating that semen did not falsely enhance the detection of HIV-1 RNA (data not shown). Although in this study we have not compared viral load measured by NASBA and by quantitative culture, the concentration of HIV-1 RNA in seminal plasma measured by NASBA has been shown to correlate well with the infectious viral titer (2), indicating that RNA levels may reflect the infectiousness of semen and seminal plasma. In other reported studies, the level of HIV-1 RNA in plasma measured by b-DNA assay and QCPCR assay has been correlated with the infectious HIV-1 titer (10, 22).

Prior reports of low viral load in seminal fluid have prompted many investigators to suggest that sexual transmission of HIV-1 occurs predominantly by exposure to seminal cells rather than to cell-free virus in seminal plasma (15, 16). This is in contrast to the observation that infection of monkeys by vaginal inoculation of cell-free SIV is 10,000-fold more efficient than infection by cell-associated virus (20). Our results showing high virus load in seminal plasma support the concept that, like transmission of SIV, transmission of HIV-1 can readily occur via cell-free virus.

The data presented here indicate that semen contains a substantially higher viral load at all levels of $CD4^+$ T cells in blood than previously recognized. These results suggest that individuals at any disease stage can potentially transmit HIV-1. Furthermore, seminal viral load can increase severalfold during progression of disease. In addition, this study shows that semen provides a readily accessible measure of viral load in an extravascular compartment, namely, the male reproductive tract. Recent reports of differential expression of quasispecies of HIV-1 in blood and semen (32) further support the importance of this site to our understanding of the biology of HIV-1 infection.

Treatment of HIV-1-infected individuals with potent protease inhibitors, such as indinavir, can lower the blood plasma viral load by more than 99% (26, 27). Whether protease inhibitors or other antiretrovirals are similarly effective in lowering viral load in the male reproductive tract was still open to question. Our data from the six subjects treated with indinavir monotherapy or a combination of indinavir and DMP-266 clearly indicate that these therapies can promptly reduce viral load in semen in parallel with that in blood plasma. However, we do not have any information on infectious viral titers or proviral DNA in seminal mononuclear cells from subjects on therapy. Further studies are needed to determine the effect of antiretroviral therapy on transmission of HIV-1. This is the first demonstration that antiviral therapy reduces HIV-1 RNA load in semen, which may be important in potential eradication of HIV-1 in body compartments other than the lymphoid system.

In summary, semen from HIV-1-infected men contains higher levels of HIV-1 RNA than previously recognized. Viral load in semen is strongly correlated with that in blood plasma after the first few years of infection, but not with the blood $CD4^+$ T-cell count. Potent antiretroviral therapy with protease and nonnucleoside RT inhibitors can rapidly reduce viral load in semen and blood. These findings have important implications for the biology of sexual transmission of HIV-1 and its potential reduction by antiretroviral therapy. At present, however, we do not have evidence that HIV-1 transmission from the subjects treated with antiretroviral therapy is decreased.

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