Differential Cellular Distribution of HIV-1 Drug Resistance in Vivo: Evidence for Infection of CD8+ T Cells during HAART

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This study presents a detailed analysis of HIV-1 populations isolated from total PBMC, plasma, CD4+ T cells, CD8+ T cells, and monocytes/macrophages in 13 patients receiving HAART. Sequence analysis of the reverse transcriptase and protease genes indicated that viral strains isolated from different blood leukocytes were genetically distinct in each subject. Notably, HIV variants isolated from CD8+ T cells were distantly related to strains derived from other blood cell types, providing evidence for the strain-specific infection of CD8+ T cells in vivo. Compartmentalization of drug resistance mutations in specific blood cell types was observed in approximately 50% of patients. The prevalence of resistance mutations was higher in either CD4+ T cells or monocytes/macrophages in these subjects. However, CD8+ T cells showed markedly lower levels of viral drug resistance in these patients, indicating a lack of viral replication in this compartment. This study is the first to demonstrate the differential distribution of HIV drug resistance in different blood cell types during HAART and provides new insights into the infection of CD8+ T cells in vivo. © 2003 Elsevier Science (USA)

Key Words: HIV-1; HAART; HIV-1 drug resistance; compartmentalization; CD8+ T cells.

INTRODUCTION

The introduction of highly active antiretroviral therapy (HAART) has led to a remarkable decline in HIV-associated morbidity and mortality in the developed world (Gulick *et al.,* 1997; Hammer *et al.,* 1997; Palella *et al.,* 1998). A significant proportion of HIV-infected individuals receiving HAART develop undetectable plasma HIV-1 viral RNA levels (<50 copies/ml) (Hirschel and Opravil, 1999). However, the emergence of drug-resistant HIV-1 is an increasing limitation of antiretroviral therapy (Hirsch *et al.,* 1998). Of major concern is the rise of HIV-1 variants resistant to multiple antiretroviral agents which are as fit as or in some cases fitter than wild type virus (Caliendo *et al.,* 1996; Kosalaraksa *et al.,* 1998). In addition, persistently infected, nonactivated T-lymphocytes have been demonstrated in individuals receiving HAART with undetectable viral RNA in blood plasma (Wong *et al.,* 1997a). Latent viral reservoirs of this kind may reseed the body and hinder attempts at HIV eradication.

During treatment with HAART, the selection of variants with drug resistance mutations is determined by a balance of the selective advantages conferred by these mutations in the presence of antiretroviral drugs and their impact on replicative capacity. The independent evolution of resistant variants in different anatomical compartments, especially during the implementation of multiple drugs with different pharmacokinetic parameters, may have a strong influence on the eventual outcome of HAART treatment. Several studies have shown that drug concentrations *in vivo* can vary considerably from one tissue type to another during antiretroviral treatment (Kepler and Perelson, 1998; Wong *et al.,* 1997a). In addition, some compartments have been shown to be poorly accessible by different antiretroviral drugs, including cerebrospinal fluid (Hoetelmans, 1998), genital tract secretions (Overbaugh *et al.,* 1996), and lymphoid tissue (Omrani and Pillay, 2000).

Although differences in the composition of viral populations recovered from plasma and peripheral blood mononuclear cells (PBMC) have been described (Romano *et al.,* 1999; Simmonds *et al.,* 1991; Wang *et al.,* 2000), little is known about the molecular nature of viral populations within individual blood cell types during HAART. CD4+ T cells are the principal target of HIV; however, infection of other hematopoietic cells, including CD8+ T cells (Livingstone *et al.,* 1996; Yang *et al.,* 1998; Saha *et al.,* 2001; Imlach *et al.,* 2001; McBreen *et al.,* 2001), monocytes (McElrath *et al.,* 1991; Sonza *et al.,* 2001; Lambotte *et al.,* 2000; Zhu *et al.,* 2002) and macrophages (Collman *et al.,* 1989; Gendelman and Meltzer, 1989; Englund *et al.,* 1995; Orenstein *et al.,* 1997), has

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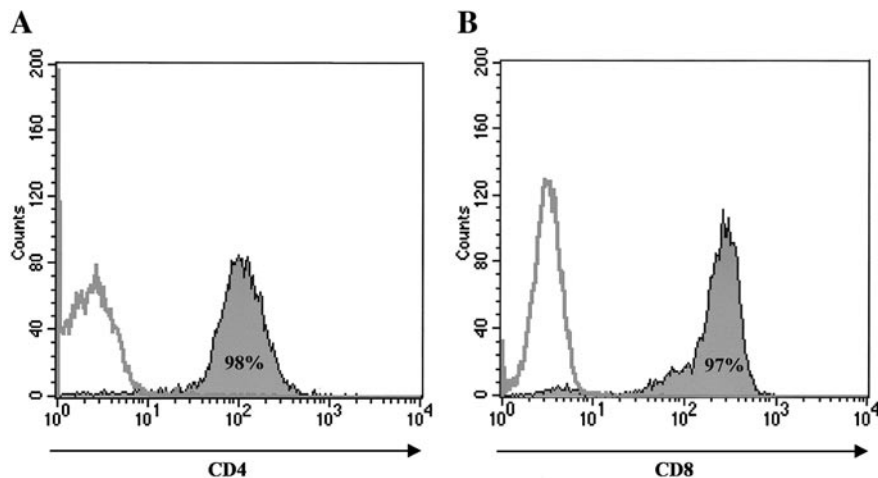
FIG. 1. FACS analysis of cells purified by positive selection from total PBMC of patient 1. (A) CD4+ T cell fraction demonstrating 98% purity. (B) CD8+ T cell fraction demonstrating 97% purity.

also been demonstrated. Differential selective pressures on viral strains within specific blood cell types may influence the emergence of drug-resistant variants. The identification of cell types that serve as drug-resistant reservoirs is crucial for the long-term success of antiretroviral treatment strategies. In addition, a reduction in the selective pressures that influence the emergence of drug-resistant viral strains in a given compartment may result in the predominance of wild-type virus capable of reseeding the viral population during the suppression of plasma viremia.

To investigate these features of HIV infection, we have conducted a detailed analysis of HIV-1 populations isolated from plasma, total PBMC, CD4+ T cells, CD8+ T cells, and monocytes/macrophages in 13 patients receiving HAART, with particular emphasis on the cellular distribution of drug-resistant viral strains *in vivo*. Mutations in the protease (PR) and reverse transcriptase (RT) genes of virus from each compartment were analyzed to identify cellular reservoirs of HIV-1 drug resistance and to clarify the trafficking of viral strains between cell-free and cell-associated blood compartments. The infection of CD8+ T cells by HIV is of particular importance as antiviral responses elicited by these cells are vital to the long-term success of HAART. Therefore, we also sought to investigate the nature of viral strains derived from this blood cell compartment and to further clarify the circumstances of CD8+ T cell infection during HAART.

RESULTS

Purity of cell separations

The effectiveness of the cell separation method was assessed by fluorescence-activated cell sorting (FACS) analysis. CD4+ T cells and CD8+ T cells obtained by positive isolation were detached from the magnetic beads and left to recover in culture overnight. Subse-quent FACS analysis revealed that contamination by other cell types in each fraction was very low. Figure 1 shows purity data obtained from analysis of separated cell fractions derived from patient 1. The CD4+ T cell fraction contained only 2% of cells that did not express the CD4 marker. In the CD8+ T cell fraction only a minimal percentage of cells (3%) did not express the CD8 marker. The purity of the monocyte/macrophage cell fraction obtained by positive isolation could not be assessed by FACS analysis because it was not possible to detach cells from the beads. However, the purity of each T cell fraction strongly supports the effectiveness of this methodology. Analysis of proviral DNA extracted from each cell fraction also provided evidence that separations were effective. In a number of patients the antiretroviral drug resistance mutations present in virus derived from each cell type were notably different, indicating that they were derived from a different source (Table 2). In subjects with a homogeneous distribution of drug resistance mutations in each compartment, cell-specific variations that were unrelated to drug-resistance were observed in the protease and reverse transcriptase peptide sequences (Table 3). Preliminary analysis of viral envelope sequences also indicated that viral populations obtained from different cell compartments were distinct (data not shown).

Compartmentalization of drug-resistant viral strains in different blood cell types occurs in some patients during HAART

HIV-1 protease and reverse transcriptase gene sequences derived from different blood compartments (plasma, total PBMC, CD4+ T cells, CD8+ T cells, and monocytes/macrophages) were analyzed for drug resistance mutations in 13 patients (Table 2). In 3 of 13 patients, PCR amplification of virus from the CD8+ T cell

TABLE 1

Clinical Features of Study Participants

Patient	T-cell count (/μl)			Viral load (copies/ml)	Antiretroviral drug history and year commenced
	CD4+	CD8+	CD4:CD8		
1	8 (2%)	172 (43%)	0.047	>100,000	1991: AZT, 3TC, ddI, IDV, d4T, ABV, SQV, EFV, NFV, RTV, LPV
2	14 (2%)	420 (60%)	0.034	>100,000	1992: AZT, ddI, 3TC, d4T, SQV, ABV, RTV, IDV
3	200	790	0.25	>100,000	1996: SQV, ddC, IDV, RTV, SQV, NVP, ddI, 3TC, NFV, ABV, d4T
4	16 (1%)	n/a		>87,700	1992: ZDV, 3TC, IDV, NFV, NVP, d4T, SQV, RTV, ddI, ABV, EFV, APV, LPV
5	60	900	0.067	>100,000	1998: d4T, AZT, SQV
6	580	870	0.67	<400	1996: d4T, SQV, ddC, 3TC, IDV, NFV, NVP, APV, ddI
7	320 (20%)	800 (50%)	0.4	60,200	1991: EFV, IDV, d4T, 3TC, ddC, ZDV, ddI, ABV, LPV
8	195 (13%)	1110 (74%)	0.18	1,510	1991: AZT, ddI, ddC, 3TC, d4T, NFV, NVP
9	135 (5%)	1647 (61%)	0.08	64,500	1990: ZDV, 3TC, SQV, ddI, NFV, RTV, d4T
10	360 (36%)	460 (46%)	0.78	<400	1995: ZDV, ddI, IDV, EFV, NFV
11	1012 (22%)	2806 (61%)	0.36	<400	1995: ZDV, ddI, 3TC, IDV, d4T, NVP
12	105 (7%)	675 (45%)	0.16	<400	1997: ZDV, 3TC, AZT, d4T, ddI, SQV, RTV, EFV
13	437 (23%)	950 (50%)	0.46	5,730	1994: ddI, AZT, 3TC, ddC, SQV, d4T, ddI, NVP, NFV

Note. n/a, not available.

and monocyte/macrophage fractions was unsuccessful. The plasma viral load of these patients was either low (patient 13) or below detectable level (<400 copies/ml) (patients 11 and 12) which suggested a reduction in the prevalence of CD8+ T cell and/or monocyte infection in these viral load categories. Monocyte/macrophage cell fractions were not isolated from patients 6 and 7 due to paucity of samples.

The distribution of drug resistance mutations in different blood compartments was similar in some patients and variable in others. In 5 subjects (patients 1, 2, 5, 6, and 9) drug resistance mutations in virus from each compartment were almost or completely identical. However, cell-specific variations in the protease and reverse transcriptase peptide sequences that were not related to drug resistance were observed in these individuals (Table 3). These patients had a range of plasma viremia including high (>100,000 copies/ml: patients 1, 2, and 5), intermediate (64,500 copies/ml: patient 9), and below detection (<400 copies/ml: patient 6) viral loads. CD4+ T cell counts ranged from very low (10/μl blood: patient 1) to normal (520/μl blood: patient 6).

In contrast to the patients above, the profile of drug resistance mutations in virus from different blood compartments was notably different in other subjects. Patients 3, 4, 7, 8, and 10 provided evidence that distinct viral populations harboring specific drug resistance mutations were present in different blood compartments. These patients had a range of plasma viremia and CD4+ T cells counts (Table 1). In general, the compartmental variation in protease drug resistance mutations was less than that of the reverse transcriptase gene. The most notable feature in each of these 5 patients was that virus derived from the CD8+ T cell compartment displayed a marked reduction in resistance to protease and/or re-

verse transcriptase inhibitors. In patients 3, 4, and 10 a number of drug resistance mutations present in the RT region of virus from the CD4+ T cell and monocyte/macrophage fractions were absent in virus derived from the CD8+ T cell compartment (Table 2). Similarly, resistance to both PR and RT inhibitors in CD8+ T cells was lower than that of the CD4+ T cell and/or monocyte/macrophage fractions in both patients 7 and 8 (Table 2).

The monocyte/macrophage fraction was the most drug-resistant blood cell compartment in patients 3 and 8. Several resistance mutations present in the PR (L10I/L) and RT (A62A/V and G190A) genes of the monocyte/macrophage and plasma fractions in patient 3 were absent in virus from the CD4+ and CD8+ T cell compartments. Resistance to RT inhibitors was similar in virus from CD4+ T cells and monocytes/macrophages in patient 8; however, several PR resistance mutations (K20V, M36I, A71I, and L90M) detected in monocytes/macrophages and plasma were absent in the CD4+ and CD8+ T cell fractions. In contrast to patients 3 and 8, the CD4+ T cell fraction was the most drug-resistant blood cell compartment in patients 4 and 10. Additional mutations in virus from the CD4+ T cell fraction increased the level of resistance to both PR and RT inhibitors compared to other cellular compartments in patient 4 (Table 2). In patient 10, resistance to PR inhibitors was identical in all three blood cell fractions but CD4+ T cells contained more resistance mutations to RT inhibitors (Table 2).

The data above indicate that although cellular compartmentalization of drug-resistant viral strains was evident in 5 of 13 patients, no clear pattern of cell-dependent accumulation of resistance was evident in these subjects. Virus derived from CD4+ T cells harbored more drug resistance mutations in some patients, while

TABLE 2

Comparison of HIV-1 Drug Resistance Mutations in Total PBMC to Those of Viral Populations from CD4^{+} T Cells,
CD8^{+} T Cells and Monocytes/Macrophages

Patient	Source	Protease resistance mutations	Change(s) in drug resistance compared to total PBMC	Reverse transcriptase resistance mutations	Change(s) in drug resistance compared to total PBMC
1	PBMC	L10I, K20I, M36I, M46I, L63P, A71V, G73S, I84V, L90M, I93L		M41L, E44D, D67N, L74V, L100I, K103N, V118I, L210W, T215Y, K219R	
	CD4	Identical to PBMC		Identical to PBMC	
	CD8	L10I → L10V	No change	Identical to PBMC	
	Monocytes	Identical to PBMC		Identical to PBMC	
	Plasma	Identical to PBMC		Identical to PBMC	
2	PBMC	L10I, L63P, A71V, G73S, V77I, L90M, I93L		M41L, D67E, T69S_V/T, L74I/V, V108I, V179I, Y181C, L210W, T215Y	
	CD4	Identical to PBMC		L74I/V → L74V	(−) AZT/d4T
	CD8	Identical to PBMC		L74I/V → L74I	(−) AZT/d4T
	Monocytes	Identical to PBMC		L74I/V → L74V	(−) AZT/d4T
				Additional mutation: K101Q	(+) All NNRTI
	Plasma	Additional mutation: K20I/K	(+) All PR	L74I/V → L74I/V	No change
3	PBMC	K20V, M46I/M, L63P, A71V, G73S, V77I, I84V, L90M		A62A/V, L74L/V, V75I/V, F77F/L, K103K/N, Y115F/Y, F116F/Y, Q151K/L/M/Q, Y181C, M184V, G190A/G	
	CD4	Identical to PBMC		Y181C → Y181C/Y	(+) AZT
				Absent mutations: A62A/V	(−) All NRTI (except 3TC)
				G190A/G	(−−−) EFV/NVP
	CD8	M46I/M → M46I/L	No change	L74L/V → L74V	(−) AZT, d4T
				K103K/N → K103N, Y115F/Y → Y115F	No change
				Absent mutations: A62A/V	(−) All NRTI (except 3TC)
				V75I/V	(−) All NRTI [(−−) d4T]
				F77F/L, F116F/Y	(−) All NRTI
				Q151K/L/M/Q	(−−−) All NRTI [(−−) ABC (−) 3TC/TDF]
				Y181C	(−−−) DLV/NVP (−−) EFV (+) AZT
				G190A/G	(−−−) EFV/NVP
	Monocytes	K20V → K20E/K/M/V	No change	G190A/G → G190A	No change
		Additional mutation: L10I/L	(+) All PR		
	Plasma	M46I/M → M46I	No change	V75I/V → V75I, F77F/L → F77L, Y115F/Y → Y115F	No change
		Additional mutation: L10I/L	(+) All PR	F116F/Y → F116Y, Q151K/L/M/Q → Q151M	No change
				Additional mutations: V108I/V	(+) All NNRTI
				Absent mutations: L74L/V	(−−−) ddI/ddC (−−) ABC
				K103K/N	(−−−) All NNRTI
4	PBMC	L10F, L24I, M36I, M46I, I54V, L63P, V82A		D67N, K70K/R, L74V, A98G, K103N, Y115F, Q151M/Q, Y181C, M184M/V, G190A/G, T215F/I/S/T, K219Q	
	CD4	Additional mutations: K20K/R	(+) All PR	D67N → D67D/G/N/S	(+) ddI/ddC
		L90L/M	(+++) NFV (++) IDV/RTV/SQV	L74V → L74L/V	(+) AZT/d4T
			(+) APV/LPV	Y115F → Y115F/Y	No change
				Y181C → Y181C/W	(+) AZT
				Additional mutations: V75I/V	(+) All NRTI [(++)d4T]
	CD8	Identical to PBMC		Y115F → Y115F/Y	No change
				Absent mutations: K70K/R	(−−) AZT (−) TDF
				M184M/V	(−−−) 3TC (−−) ddI/ddC/ABC
				G190A/G	(−−−) EFV/NVP
				Q151M/Q	(−−) All NRTI [(−) 3TC/TDF]
	Monocytes	Identical to PBMC		K70K/R → K70R, Q151M/Q → Q151M, G190A/G → G190A, T215F/I/S/T → T215F	No change
				K103N → K103H	(−−) All NNRTI
				M184M/V → M184V	(−−) AZT/d4T/TDF
	Plasma	M46I → M46I/L	No change	K219Q → K219K/Q	No change
		Additional mutations: K20K/R	(+) All PR	Additional mutations: M41I/M	(+) AZT
				Absent mutations: K70K/R	(−−) AZT (−) TDF
				Y115F	(−) ABC
				Q151M/Q	(−−) All NRTI [(−) 3TC/TDF]
				M184M/V	(−−−) 3TC (−−) ddI/ddC/ABC
				G190A/G	(−−−) EFV/NVP
5	PBMC	L63A		K103K/N, V179I	
	CD4	Identical to PBMC		Identical to PBMC	
	CD8	Identical to PBMC		Identical to PBMC	
	Monocytes	Identical to PBMC		Identical to PBMC	
	Plasma	Identical to PBMC		Identical to PBMC	
6[a]	PBMC	L63P		None	
	CD4	Identical to PBMC		Identical to PBMC	
	CD8	Identical to PBMC		Identical to PBMC	
	Plasma	Identical to PBMC		Identical to PBMC	

TABLE 2—*Continued*

Patient	Source	Protease resistance mutations	Change(s) in drug resistance compared to total PBMC	Reverse transcriptase resistance mutations	Change(s) in drug resistance compared to total PBMC
7[a]	PBMC	L10F/L, V32I/V, M46L/M, L63L/P, A71A/V, V82A/V		M41L, D67N, A98A/G, K103N, V118I/V, M184V, L210W, T215Y	
	CD4	L63L/P → L63P	No change	Identical to PBMC	
		Absent mutation: V82A/V	(−−) IDV/NFV/RTV (−) APV/SQV/LPV		
	CD8	Absent mutations: V32I/V	(−) All PR [(−−) APV]	Absent mutations: A98A/G	(−) All NNRTI
		L10F/L, L63L/P, A71A/V	(−) All PR	V118I/V	(−−−) All NNRTI
		M46L/M	(−) All PR [(−−) NFV]		
		V82A/V	(−−) IDV/NFV/RTV (−) APV/SQV/LPV		
	Plasma	L10F/L → L10F, V32I/V → V32I	No change	A98A/G → A98G, V118I/V → V118I	No change
		M46L/M → M46L, L63L/P → L63P	No change	Additional mutations: L74I	(+++) ddI/ddC (++) ABC (−) AZT/d4T
		A71A/V → A71V, V82A/V → V82A	No change	V108I	(+) All NNRTI
8	PBMC	L63L/P		M41L/M, E44D/E, A98S, K103K/N, M184M/V, L210W, T215N/S/T/Y	
	CD4	Identical to PBMC		M41L/M → M41L, T215N/S/T/Y → T215Y	No change
				Absent mutation: E44D/E	(−) 3TC
	CD8	L63L/P → L63P	No change	M41L/M → M41L, E44D/E → E44D	No change
				K103K/N → K103N	No change
				M184M/V → M184V	(−) AZT/d4T/TDF
				T215N/S/T/Y → T215S	(−) All NRTI [except 3TC]
	Monocytes	L63L/P → L63P	No change	M41L/M → M41L, K103K/N → K103N	No change
		Additional mutations:		T215N/S/T/Y → T215Y	No change
		K20V, M36I, A71I	(+) All PR	M184M/V → M184V	(−) AZT/d4T/TDF
		L90M	(+++) NFV (++) SQV/RTV/ITV (+) APV/LPV	Absent mutation: E44D/E	(−) 3TC
	Plasma	L63L/P → L63P	No change	M41L/M → M41L, K103K/N → K103N	No change
		Additional mutations:		T215N/S/T/Y → T215Y	No change
		K20V, A71V, G73S	(+) All PR	M184M/V → M184V	(−) AZT/d4T/TDF
		L90M	(+++) NFV (++) SQV/RTV/ITV (+) APV/LPV	Absent mutation: E44D/E	(−) 3TC
9	PBMC	K20R, D30N, M36I, L63P, A71T, N88D		M41L, E44A, D67N, K103N, V118I, Y181C, L210W, T215Y, K219N	
	CD4	Identical to PBMC		Identical to PBMC	
	CD8	Identical to PBMC		Identical to PBMC	
	Monocytes	Identical to PBMC		Identical to PBMC	
	Plasma	Identical to PBMC		Identical to PBMC	
10	PBMC	L63P		M41L/M, L210L/W, T215N/S/T/Y	
	CD4	Identical to PBMC		Identical to PBMC	
	CD8	Identical to PBMC		Absent mutations: M41L/M, L210L/W	(−) All NRTI (except 3TC)
				T215N/S/T/Y	(−−) AZT/d4T/ABC (−) ddI/ddC/TDF
	Monocytes	Identical to PBMC		Additional mutation: K70K/R	(++) AZT (+) TDF
				Absent mutations: M41L/M, L210L/W	(−) All NRTI (except 3TC)
				T215N/S/T/Y	(−−) AZT/d4T/ABC (−) ddI/ddC/TDF
11[b]	PBMC	L10I, M36I, L63P, V77I, I93L		M184M/V	
	CD4	Identical to PBMC		Additional mutation: K103K/R	(+) All NNRTI
	Plasma	Absent mutation: M36I	(−) All PR	Absent mutation: M184M/V	(−−−) 3TC (−−) ddI/ddC/ABC
				Additional mutation: K103K/N	(+++) All NNRTI
12[b]	PBMC	None		K103R	
	CD4	Additional mutation: D30N	(+++) NFV	Additional mutation: M230I	(+) All NNRTI
	Plasma	Additional mutations: M46I/M	(++) NFV/APV (+) IDV/SQV/LPV	Additional mutations: K101E	(+) All NNRTI
		L63P	(+) All PR	V108I/V	(+) All NNRTI
13[b]	PBMC	L63A/P/S, V77I, I93I/L		A98S	
	CD4	Identical to PBMC		Additional mutations: D67D/N, K219K/Q	(++) AZT (+) ddI/ddC/d4T/ABC/TDF
				T215S/T	(++) AZT (+) d4T/ABC/TDF
	Plasma	L63A/P/S → L63A	No change	Additional mutations: D67N, K219Q	(++) AZT (+) ddI/ddC/d4T/ABC/TDF
		Additional mutations: L10V, I54V	(+) All PR	K70R	(++) AZT (+) TDF
		G48V	(+) All PR [(+++) SQV (++) NFV]	T215F	(++) AZT/d4T/ABC (+) ddI/ddC/TDF
		V82A	(++) IDV/NFV/RTV (+) APV/SQV/TD		
		Absent mutation: I93I/L	(−) All PR		

Note. Nomenclature of drug resistance mutations: (i) single letter abbreviation preceding number indicates identity of wild-type/consensus amino acid at given position; (ii) number represents position of amino acid in peptide chain of RT or PR enzyme; (iii) single letter abbreviation(s) following number indicates substitute amino acid in mutant virus at given position. (+), small increase; (++), medium increase; (+++), large increase in the level of resistance to specified antiretroviral drug in comparison to the general PBMC viral population; (−), small decrease; (−−), medium decrease; (−−−), large decrease in the level of resistance to specified antiretroviral drug in comparison to the general PBMC viral population.

[a] Isolation of monocyte/macrophage cell fraction not performed due to paucity of sample.

[b] PCR amplification of virus in CD8+ T cell and monocyte/macrophage cell fractions unsuccessful.
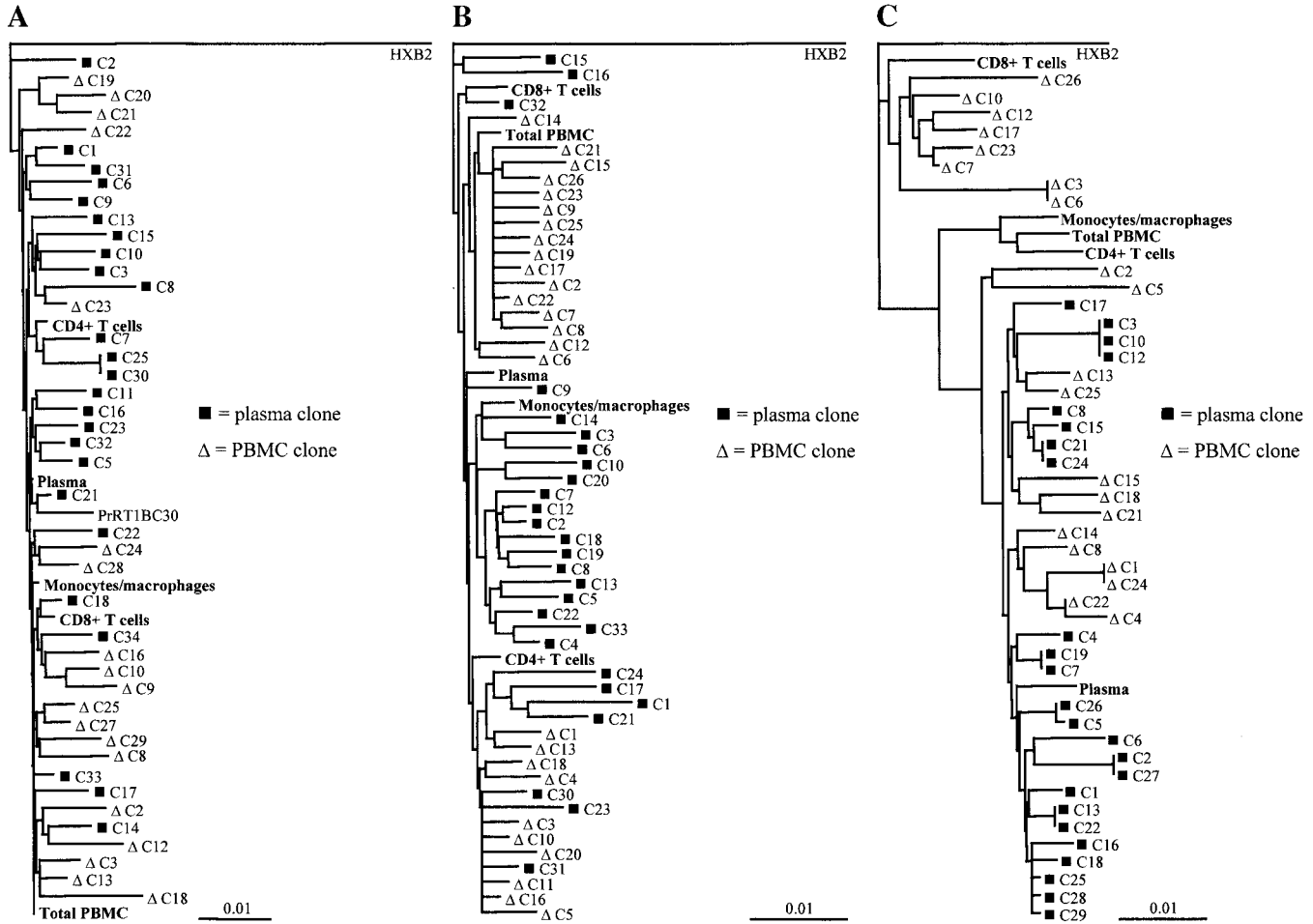
FIG. 2. Distance neighbor-joining trees showing phylogenetic relationships between RT-PR nucleotide sequences derived from total PBMC viral clones (△), plasma viral clones (■), and the major viral population from various blood cell types (CD4+ T cells, CD8+ T cells, and monocytes/macrophages) in patients 1–4 (A–D) and patient 11 (E).

monocytes/macrophages showed more drug resistance in others. Nonetheless, it is clear that multiple blood cell types harbored drug-resistant viral strains in these patients with the notable exception of CD8+ T cells in several cases.

Trafficking of drug-resistant viral strains between cell-free and cell-associated viral compartments during HAART: Analysis of viral clones from the plasma and total PBMC viral populations

Clonal analysis of plasma and total PBMC viral populations was performed in 5 of 13 patients. The RT-PR genes were sequenced in 20–25 viral clones from each compartment. To best represent the group as a whole, patients selected for this analysis had a range of different characteristics including variable plasma viremia and CD4+ T cell counts. The pattern of viral drug resistance in different blood compartments was very similar in 2 of these patients (patients 1 and 2), but variable in others (patients 3 and 4). Patient 11 was representative of several subjects in which PCR amplification of proviral

DNA from CD8+ T cells and monocytes/macrophages was unsuccessful.

The distribution of drug resistance mutations in virus from different blood compartments was almost identical in patients 1 and 2 (Table 2). Similarly, there was little variation in the drug resistance profile of viral clones derived from the total PBMC and plasma compartments of these patients (data not shown). Figure 2A shows phylogenetic analysis of RT-PR sequences from viral clones in the total PBMC and plasma compartments of patient 1. Clonal sequences from both compartments shared a high degree of genetic homogeneity. Viral clones were also closely related to sequences derived from the total viral population of each blood cell type. This provided evidence that each blood cell compartment contributed virus to the plasma and total PBMC populations in patient 1. Figure 2B shows phylogenetic analysis of RT-PR sequences from viral clones in the total PBMC and plasma compartments of patient 2. The viral clones of each compartment were also closely related to each other, although a degree of segregation was evi-
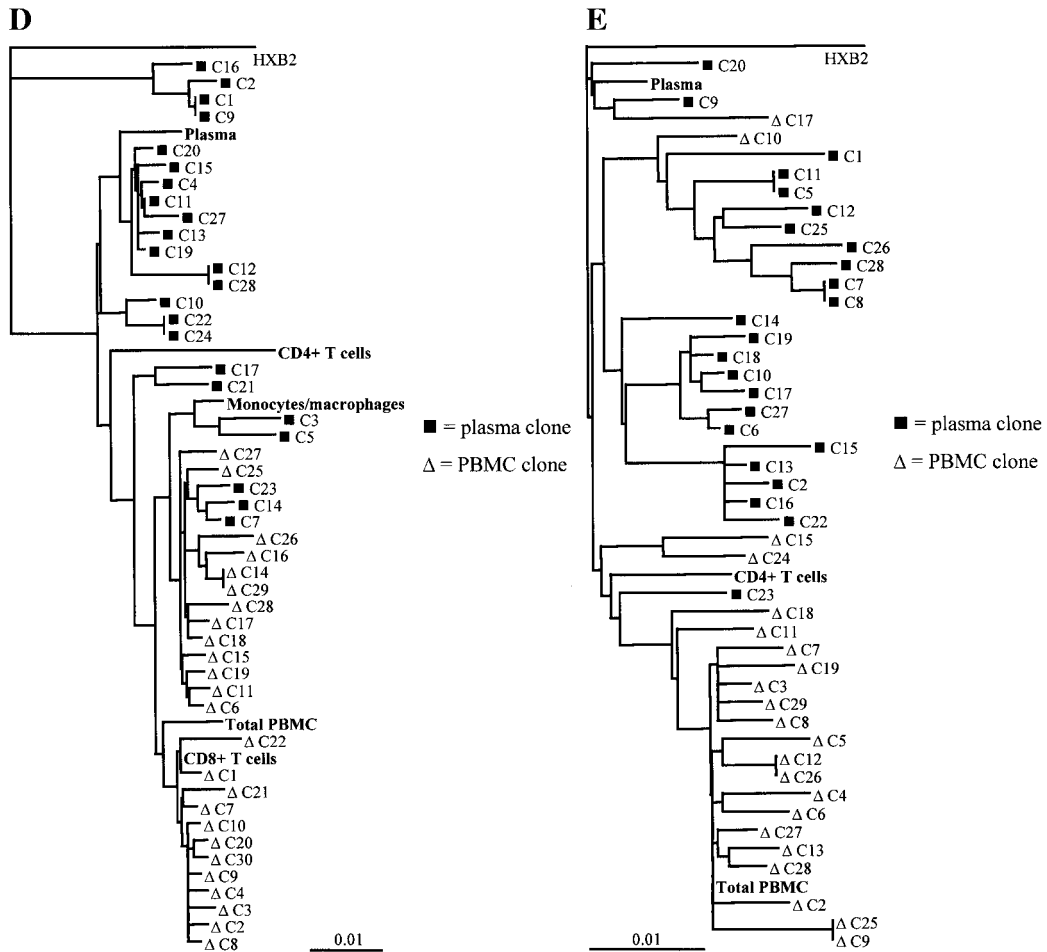
FIG. 2—*Continued*

dent. Total population sequences from CD4+ T cells and CD8+ T cells were closely associated with PBMC clones. In contrast, virus derived from the monocyte/macrophage fraction was more distantly related to PBMC clones, suggesting a reduced contribution to the total PBMC viral population. The CD4+ T cell and monocyte/macrophage viral populations were closely associated with plasma-derived clones, providing evidence of viral trafficking between these compartments. However, there was less evidence of trafficking between CD8+ T cells and plasma in patient 2.

Patients 3 and 4 showed significant variation in the drug resistance profile of viral clones derived from both the total PBMC and plasma compartments (data not shown). This correlated well with earlier data which showed heterogeneity in the drug resistance profiles of different blood compartments in these patients (Table 2). In patient 3, eight viral clones from the total PBMC compartment lacked a number of mutations conferring resistance to both PR (M46I) and RT (V55I, F77L, Y115F, F116Y, Q151M, M184V) inhibitors. These mutations were present in virus isolated from the CD4+ T cell and monocyte/macrophage compartments but absent in vi-

rus from the CD8+ T cell fraction (Table 2). Figure 2C shows that this subset of eight clones was closely related to virus from CD8+ T cells but phylogenetically distinct from monocyte/macrophage and CD4+ T cell total population sequences. This indicated that CD8+ T cells contributed variants with reduced levels of drug resistance to the total PBMC viral population. However, virus from CD8+ T cells was phylogenetically distinct from all plasma viral clones in patient 3, suggesting a minimal amount of viral trafficking between these compartments (Fig. 2C). Sequences from CD4+ T cells and monocytes/macrophages were more closely associated with plasma viral clones, providing evidence for the trafficking of drug-resistant variants (Fig. 2C).

In patient 4, viral clones from total PBMC clustered into two different groups (Fig. 2D). Clones in the upper group were closely related to virus derived from the monocyte/macrophage and CD4+ T cell compartments. Clones in the lower group were related to virus from CD8+ T cells and lacked three primary RT resistance mutations present in clones from the upper group (Q151M, M184V, and G190A). These data suggest that individual cellular compartments contributed distinct HIV

variants with characteristic profiles of drug resistance to the total PBMC viral population. Virus derived from CD8+ T cells was less closely related to plasma viral clones than virus from the monocytes/macrophage and CD4+ T cell compartments (Fig. 2D). This suggested a reduced amount of trafficking between the CD8+ T cell and plasma viral populations.

In patient 11, PCR amplification of proviral DNA from CD8+ T cells and monocytes/macrophages was unsuccessful (Table 2). This indicated a reduction in the cellular sources of virus in total PBMC. As a consequence, the degree of variation in the drug resistance profile of individual viral clones from the total PBMC compartment was reduced in patient 11 (data not shown). Fifteen of 21 clones had identical drug resistance profiles. In contrast, there was a larger degree of variation in the drug resistance profiles of viral clones from plasma (data not shown). This suggests that in addition to virus derived from total PBMC, other sources of drug-resistant virus may have contributed to the plasma viral population of this patient. This was supported by the notable degree of segregation evident between PBMC and plasma-derived viral clones (Fig. 2E).

Analysis of viral clones from the plasma and total PBMC compartments demonstrated the trafficking of drug-resistant viral strains between individual blood cell types and the plasma compartment in each of these patients. These data also indicated that CD8+ T cell infection was productive in some patients (patients 1 and 2) but may be quiescent in other subjects where CD8+ T cell variants were more distantly related to plasma viral clones (patients 3 and 4). A variable degree of compartmentalization between cell-free and cell-associated virus was observed in these five patients (Figs. 2A–2E). The segregation of viral strains from the plasma and PBMC compartments evident in some individuals was confirmed by phylogenetic analyses of total viral population sequences (Figs. 3A–3J). These findings indicate that while there was evidence of trafficking between the plasma and PBMC compartments in all subjects, the sources of cellular and plasma virus were variable in some cases and may be related to the immunological state of the patient.

HIV-1 strains isolated from CD8+ T cells are phylogenetically distinct from strains in other blood cell types: Direct evidence for strain-specific selection in CD8+ T cells *in vivo*

Phylogenetic analysis of RT-PR sequences from patients 1–10 revealed that viral populations from individual blood cell compartments were largely distinct from each other and from their plasma counterparts (Fig. 3). This was most evident in patients who displayed cellular compartmentalization of drug resistance mutations (patients 3, 4, 7, 8, and 10). Most notably, virus isolated from

CD8+ T cells was phylogenetically distinct in a number of subjects, and branched separately from other blood cell types (patients 1, 2, 3, 6, 8, and 10). In addition, when CD8+ T cell strains clustered with other cell type(s) (patients 4, 5, 7, and 9), the branch lengths were different, providing evidence of molecular differences in variants harbored by CD8+ T cells (Fig. 3). These data suggest that CD8+ T cell infection was strain dependent in these patients. The relationships between viral sequences derived from other blood compartments were variable in different patients. Phylogenetic analyses were not performed in patients 11, 12, and 13 because PCR amplification of virus from CD8+ T cells and monocytes/macrophages was unsuccessful in these subjects. Each of these patients showed low to undetectable plasma viral loads with moderate to high CD4+ and CD8+ T cell counts. This provided some indication that infection of CD8+ T cells may be less likely to occur during low plasma viremia and that the maintenance of elevated T cell counts is an important factor in suppressing the infection of CD8+ T cells.

DISCUSSION

In this study we carried out a detailed investigation of HIV variants in different blood compartments with emphasis on the distribution of drug resistance, trafficking between cell-associated and cell-free viral compartments, and the infection of CD8+ T cells *in vivo* during HAART. Although the compartmentalization of HIV-1 populations in different anatomic compartments including the brain, semen, gastrointestinal mucosa, cerebrospinal fluid, plasma, and PBMC has been documented (Simmonds *et al.,* 1991; Eyre *et al.,* 2000; Smit *et al.,* 2001; Gupta *et al.,* 2000; Romano *et al.,* 1999; Poles *et al.,* 2001; Stingele *et al.,* 2001; Wang *et al.,* 2000), little is known about the molecular nature of HIV variants in different blood leukocytes during treatment with HAART. Therefore, the aims of this study were (1) to examine the distribution of drug-resistant HIV variants in different blood cell types during HAART, and to identify those cell type(s) which harbor drug-resistant viral populations; (2) to examine the trafficking of HIV variants/resistant variants between cell-free and cell-associated compartments during HAART; and (3) to investigate the nature of viral strains derived from CD8+ T cells and further clarify the circumstances of CD8+ T cell infection during HAART.

To achieve these objectives, the protease and reverse transcriptase genes of HIV-1 strains derived from plasma, total PBMC, and individual blood cell types (CD4+ T cells, CD8+ T cells, and monocyte/macrophages) were sequenced and analyzed for drug resistance mutations in 13 patients receiving HAART. We provide the first evidence of distinct viral populations with characteristic patterns of drug resistance in differ-
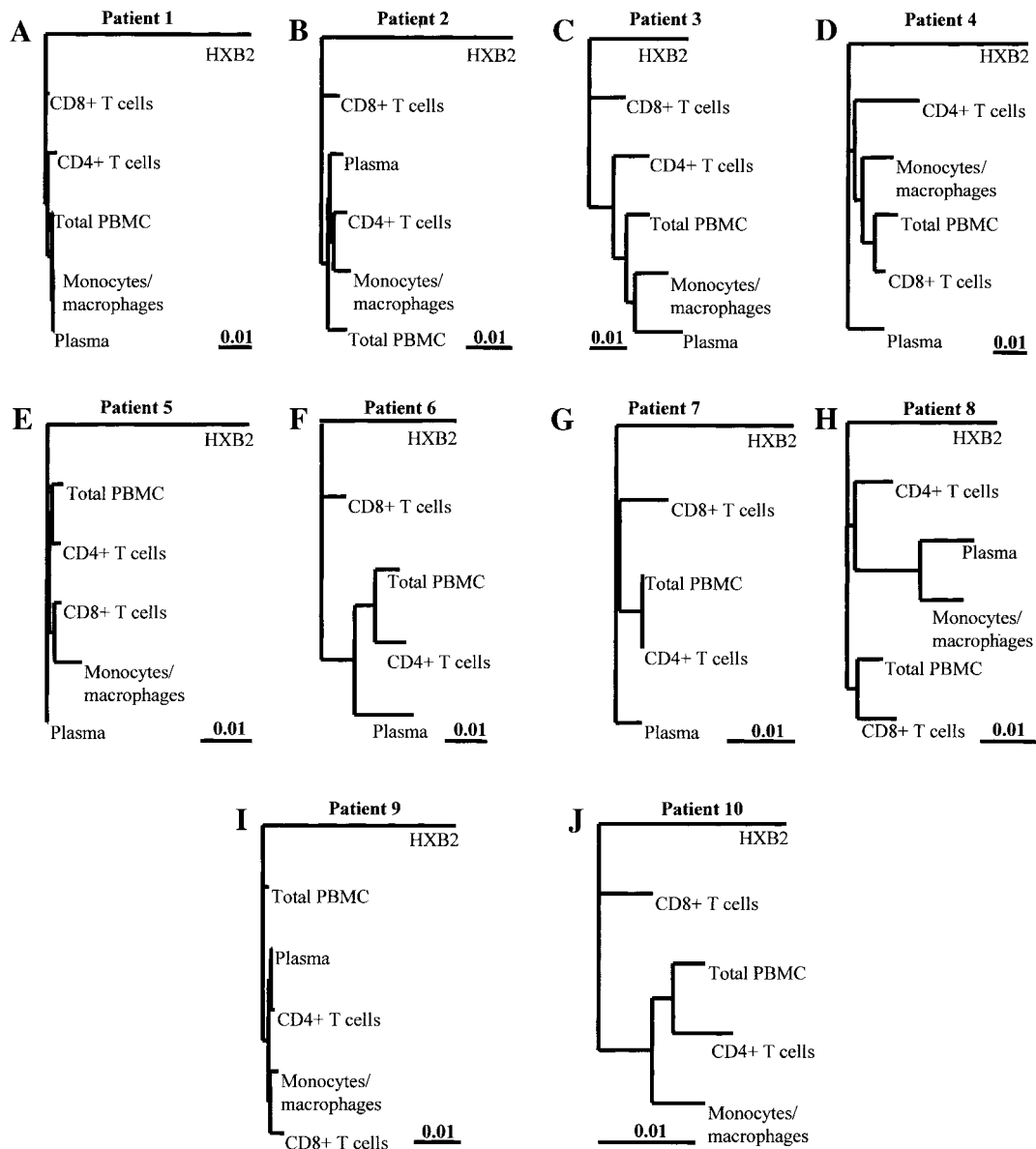
FIG. 3. Distance neighbor-joining trees showing phylogenetic relationships between the RT-protease nucleotide sequences of major viral populations from different blood compartments in patients 1–10 (A–J, respectively).

ent blood cell types during antiretroviral treatment. This study included patients with a diverse range of clinical features however, no significant correlation between compartmentalization of viral quasispecies and the level of plasma viremia or overall T cell counts was found. Drug-resistant HIV strains were isolated from patients in all viral load categories, including three in the BDL range (<400 copies/ml; patients 10, 11, and 12). Other studies have also reported the emergence of antiretroviral resistance when plasma viral load falls below 50 copies/ml during HAART (Martinez-Picado et al., 2000) and these findings are concordant with our observations.

The profile of drug resistance mutations in virus from distinct blood compartments was variable in 5 of 13 individuals. The cumulative effects of this differential distribution of drug resistance is likely to contribute to the emergence of highly resistant variants in blood during HAART. In general, there was more variation in the level of resistance to reverse transcriptase inhibitors than to protease inhibitors. The most notable feature of subjects with compartmental variation in drug resistance (patients 3, 4, 7, 8, and 10) was that viral strains derived from CD8+ T cells contained significantly less resistance mutations to protease and/or reverse transcriptase

inhibitors (Table 2). In 2 cases (patients 3 and 8), several additional mutations significantly increased the level of drug resistance in virus derived from the monocyte/macrophage compartment. These mutations were not present in CD4+ T cells or CD8+ T cells, but were observed in the plasma viral population. This suggested trafficking of drug-resistant variants between plasma and monocytes/macrophages in these patients. Analysis of viral clones from patient 3 further confirmed the occurrence of viral trafficking between these compartments (Fig. 2C). In contrast, 2 other subjects (patients 4 and 10) showed increased drug resistance in the CD4+ T cell compartment, however, the additional mutations conferring this resistance were notably absent in plasma. Our data suggest that the concordance between cell-free and cell-associated virus (both resistant and nonresistant strains) may be a random phenomenon that is dependent largely on host physiology or immunological status at any given time during HAART treatment. The pronounced segregation of plasma and total PBMC derived viral clones observed in several patients (Figs. 2C–2E) provided evidence of a differential source of plasma and PBMC viral strains in some cases. However, the independent evolution of HIV-1 strains in specific cellular compartments cannot be ruled out as a contributing element (Wong et al., 1997b).

The factors which influence the compartmentalization of viral variants during HAART and the clinical and biological consequences are not fully understood. The random nature of this compartmentalization suggests the establishment of transitory drug-resistant reservoirs in specific cell types at different stages of HAART treatment. These transitory reservoirs may facilitate the shuttling of resistant variants between different anatomical compartments and contribute to disease progression. A number of factors may influence the compartmentalization of drug resistant variants in different blood cell types during HAART. Antiviral regimens may exert selective pressures on HIV and affect the routing of viral variants between anatomical compartments (Haddad et al., 2000; Wang et al., 2000). Drug concentrations in vivo can vary considerably from one tissue type to another during antiretroviral treatment (Wong et al., 1997a; Kepler and Perelson, 1998) and some anatomical compartments are poorly accessible by different antiretroviral drugs (Overbaugh et al., 1996; Hoetelmans, 1998). Likewise, the penetration of antiretroviral drugs into different blood cells types may be variable and alter the selective pressures responsible for the emergence of drug-resistant strains. Cellular immune responses during HAART also impose selective pressures on HIV resulting in the differential distribution of independently evolving virodemes in different anatomic compartments (Wong et al., 1997b).

In contrast to the patients above, there was a uniform distribution of drug resistance in different blood compartments in a number of subjects (patients 1, 2, 5, 6, and 9). This provided evidence that virus from each blood cell compartment contributed similarly to the total PBMC and plasma populations in these subjects. Viral drug resistance in CD8+ T cells was not reduced in these five patients and was equivalent to that of virus from other blood compartments. This provided evidence for the productive infection of the CD8+ T cell compartment in these individuals. Viral clones from both the plasma and total PBMC compartments in patients 1 and 2 showed clear evidence of viral trafficking between plasma and all three blood cell compartments (Figs. 2A–2B). It is unlikely that the homogeneity of viral drug resistance observed in these patients arose from impurities in separated cell fractions. Four lines of evidence support the effectiveness of cell separations in these patients. First, FACS analysis revealed very low levels of contamination by other cell types in each cell fraction (Fig. 1). Second, sequences obtained from distinct cellular compartments (particularly those from CD8+ T cells) were notably different in many subjects, indicating that viral sequences were derived from a different source. In those patients with identical drug resistance mutations in each compartment, cell-specific variations in RT-PR peptide sequences that were unrelated to drug-resistance were still observed (Table 3). Third, preliminary analysis of viral envelope sequences indicated that viral populations obtained from different cell compartments were distinct (data not shown). Finally, the strong similarity between viral clones derived from the total PBMC and plasma viral populations of patients 1 and 2 (Figs. 2A and 2B) provided evidence that viral sequences in different blood cell compartments were homogeneous in some subjects and did not arise from impurities in separated cell fractions.

One of the primary goals of this study was to derive further information on viral strains that infect CD8+ T cells and clarify the circumstances of CD8+ T cell infection during HAART. The infection of CD8+ T cells is of particular importance because antiviral responses elicited by these cells are vital to the long-term success of HAART. There is considerable evidence that CD8+ T cells inhibit HIV-1 replication by both cytolytic and noncytolytic mechanisms (Walker et al., 1989). Chemokines secreted by CD8+ T cells including MIP-1α, MIP1-β, and RANTES play a significant role in the suppression of non-syncytium-inducing (NSI) strains of HIV in vitro by blocking viral entry via the CC-chemokine receptor CCR5 (Cocchi et al., 1995; Deng et al., 1996). Until recently, the infection of CD8+ T cells remained controversial but several studies have shown that they can be productively infected in a strain-dependent manner (Saha et al., 2001; Imlach et al., 2001). This infection may be mediated by transient expression of the CD4 receptor during the double positive stage of thymic maturation (Lee et al., 1997; McBreen et al., 2001) or subsequent to activation of the

TABLE 3

Variations in Protease/Reverse Transcriptase Peptide Sequences Not Related to Drug Resistance in Patients 1, 2, 5, 6, and 9

Patient		Plasma	PBMC	CD4+ T cells	CD8+ T cells	Macrophages
1	PR	20: K/R	20: K/R	20: K/R	20: K	20: K
	RT	177: E/K	177: E/K	177: K	177: K	177: E/K
2	PR	72: L 99: F	72: I/L 99: F	72: I 99: F	72: L 99: FS	72: L 99: F
	RT	88: C/W 122: E/Q	88: C 122: E	88: C 122: Q	88: C 122: E	88: C 122: E
5	PR	64: M 65: E	64: M 65: E	64 M: 65: E	64: I/M 65: D/E	64: I/M 65: D/E
	RT	35: V 50: I	35: I 50: I	35: I/V 50: I	35: V 50: I	35: V 50: I/S
6	PR	14: R 70: R	14: R 70: K	14: R 70: K	14: R 70: K	14: KR 70: K
	RT	202: V 206: Q	202: I 206: Q	202: I 206: Q	202: I 206: Q	202: I 206: E/Q
9	PR	13: I	13: I/V	13: I/V	13: I	13: I

Note. PR, protease; RT, reverse transcriptase. Numbers refer to position of amino acid in peptide chain of the protein indicated. Single letter abbreviation(s) following each number indicates the identity of amino acid at this position.

T cell receptor complex (Imlach *et al.,* 2001; Kitchen *et al.,* 1998; Flamand *et al.,* 1998; Yang *et al.,* 1998). These data clearly demonstrate the infection of CD8+ T cells during HAART in 10 of 13 subjects. In addition, phylogenetic analysis of reverse transcriptase and protease nucleotide sequences indicated that HIV variants isolated from CD8+ T cells were distantly related to strains derived from other blood cell types in patients 1, 2, 3, 6, 8, and 10 (Fig. 3), confirming the strain-specific infection of CD8+ T cells *in vivo.*

At present, there is no clear explanation for the notable absence of drug resistance mutations in virus derived from CD8+ T cells observed in some subjects (patients 3, 4, 7, 8, and 10). One possibility is that antiretroviral drugs were less efficient in targeting CD8+ T cells in some individuals, leading to a decrease in the selective pressure for the development of drug-resistant variants. Alternatively, certain drug-resistant strains may not be tropic for CD8+ T cell entry. It is also conceivable that these strains represent "archival species" established in memory CD8+ T cell subsets soon after primary sero-conversion. Several studies have demonstrated that latently infected memory CD4+ T cells produce low levels of virus capable of reseeding the body during successful treatment with HAART (Wong *et al.,* 1997a; Finzi *et al.,* 1997; Zhang *et al.,* 1999; Furtado *et al.,* 1999). This latent viral reservoir is established soon after infection (Chun *et al.,* 1998a) and can be activated by proinflammatory cytokines *in vitro* and potentially *in vivo* (Chun *et al.,* 1998b). Viral strains derived from latently infected CD4+ T cells have been shown to contain few antiretroviral drug resistance mutations compared to the predominant viral population, suggesting an absence of ongoing replication (Wong *et al.,* 1997a; Zhang *et al.,* 1998; Furtado *et al.,* 1999). The marked absence of drug resistance mutations in the CD8+ T cell viral population of several subjects also indicated a lack of viral replication and raises the possibility that, in some cases, memory CD8+ T cells may act as a latent viral reservoir. However, this was not confirmed in this study because the individual contribu-

tion of the memory CD8+ T cell subset was not assessed. Other mechanisms including tissue distribution, persistence, or slower replication could also account for this phenomenon. In addition, CD8+ T cell infection appeared to be productive in a number of other cases (patients 1, 2, 5, 6, and 9), further complicating this issue. Nonetheless, the findings presented here on the infection of total CD8+ T cell populations are of significant biological relevance to the clinical management of HIV patients. The protection of CD8+ T cells from HIV-1 infection during HAART will lead to better T cell responses and is crucial for long-lasting natural immunity in infected patients.

Our data suggest that CD8+ T cell infection may predominantly occur during intermediate or high levels of plasma viremia, concurrent with a decline in CD4+/CD8+ T cell counts. Subjects with higher viral loads (patients 1–5) displayed a reduction in the number of CD4+ T cells and/or CD8+ T cells (Table 1). Both cell types play a crucial role in controlling the level of plasma viremia, and this reduction may account for the infection of CD8+ T cells in these patients. Subjects with intermediate or low viral loads generally displayed higher CD8+ T cell counts. In addition, plasma viremia was low or undetectable and CD8+ T cell counts were elevated in the 3 patients from whom we failed to successfully amplify HIV in the CD8+ T cell compartment (patients 11, 12, and 13). These data suggest a correlation between high overall T cell counts and the maintenance of below detection to intermediate levels of plasma viremia during HAART. The actual mechanism of CD8+ T cell infection is uncertain, however, HIV uses an array of measures to interfere with the expression and production of essential antiviral factors produced by CD8+ T cells (Walker *et al.,* 1989). The ability of HIV to hinder the production and maturation of key cytokines and chemokines significantly contributes to deterioration of CD8+ T cell responses during high plasma viremia. This was emphasized in a case study of 20 patients in which both the number of CD8+ T cells and the degree of CD8+ T cell

antiviral activity were shown to steadily decline during prolonged HAART treatment (Imami et al., 2001) and may explain why CD8+ T cell antiviral responses are significantly reduced or lost under high viremia.

This study is the first to provide a detailed analysis of viral strains isolated from different blood cell compartments during highly active antiretroviral therapy. Our data show that distinct viral populations harboring specific drug resistance mutations were present in different blood compartments in some patients receiving HAART. These findings also emphasize that both monocytes/macrophages and CD4+ T cells may serve as reservoirs of drug-resistant viral strains during antiretroviral treatment. The cellular and biological consequences of the compartmentalization of drug resistance viral variants during HAART remain unclear and may have important implications for future design of antiretroviral treatment strategies. Few studies have addressed the HIV-1 infection of CD8+ T cells. Our data clearly demonstrated the infection of CD8+ T cells in vivo in subjects with a diverse range of plasma viremia and CD4+ T cell counts. In several patients, CD8+ T cells also showed markedly lower levels of viral drug resistance, indicating a lack of viral replication. We found clear evidence suggesting that viral infection of CD8+ T cells in vivo occurs in a strain-dependent manner, and propose that a significant correlation may exist between the infection of this compartment and the level of plasma viremia and/or overall T cell counts. In view of these findings, future studies addressing the timing, mechanisms, and effects of CD8+ T cell infection are essential to develop treatments that can assist in the recovery of CD8+ T cell antiviral responses during HAART. Further, the infection of CD8+ T cells may provide a marker to predict the long-term efficacy of HAART treatment. Although the effect of the emergence of drug-resistant HIV variants on the outcome of antiretroviral treatment is clear, the actual clinical and biological relevance of the cellular compartmentalization of drug-resistant strains remains to be established. Clarification of these aspects of infection will benefit future treatment strategies for HIV patients.

MATERIALS AND METHODS

Study population

Thirteen HIV-1 seropositive patients with a range of different plasma viremia and CD4+ T cell counts were enrolled in this study. Treatment histories and clinical profiles are shown in Table 1. A single blood sample was collected from each individual after informed consent. All subjects (with the exception of patient 5) were receiving HAART at the time of sampling. Plasma viral loads were determined by using a commercially available assay (Amplicor HIV-1 Monitor Test Version 1.0; Roche, Castle Hill, NSW, Australia).

Cell separations

Samples (10–20 ml) of whole blood anticoagulated with EDTA were obtained from each patient. Samples were diluted with an equal volume of phosphate-buffered saline (PBS) and peripheral blood mononuclear cells (PBMC) isolated by Ficoll–Hypaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). Individual blood cell types (CD4+ T cells, CD8+ T cells, and monocytes/macrophages) were separated by positive isolation using antibody-conjugated magnetic beads (Dynal Biotech, Oslo, Norway) as per manufacturer's instructions.

Fluorescence-activated cell sorting (FACS) analysis

CD4+ T cells and CD8+ T cells were separated from freshly isolated PBMC and rested overnight in culture medium (RPMI/10% FCS). Both R-phycoerythin (R-PE)-conjugated CD4 antibody (clone S3.5, 5 μl per 10^5 cells; Caltag Laboratories, Burlingame, CA) and fluorescein isothiocyanate isomer 1 (FITC)-conjugated CD8 antibody (clone 3B5, 5 μl per 10^5 cells; Caltag Laboratories) were added to each cell suspension. R-PE-conjugated mouse IgG2a antibody (clone UPC-10, 10 μl per 10^5 cells; Caltag Laboratories) and FITC-conjugated mouse IgG2a antibody (clone UPC-10, 10 μl per 10^5 cells; Caltag Laboratories) were used as isotype controls for both CD4+ T cell and CD8+ T cell fractions. After incubation for 30 min at 4°C, cells were washed twice in PBS and fixed in 200 μl of 1% paraformaldehyde. The purity of each cell population was determined using a FACScan (Becton–Dickinson, Franklin Lakes, NJ) and analyzed with CellQuest V3.3 software (Becton–Dickinson).

Nucleic acid extraction and PCR amplification

DNA was isolated using a QIAamp DNA mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was isolated from plasma using TRI reagent (Sigma, St. Louis, MO) and reverse-transcribed into cDNA with Superscript II (GIBCO/BRL, Rockville, MD) as per manufacturer's instructions. A nested polymerase chain reaction (PCR) was used to amplify a 1099-bp region of the HIV genome containing the reverse transcriptase (RT) and protease (PR) genes using primers 5′-GAAGAGACAGCAACTCCCTCTCAG-3′ (outer, sense), 5′-GCCCAATTCAATTTTCCCACTAAC-3′ (outer, antisense), 5′-GACAAGGAACTGTATCCTTTAGCTTC-3′ (inner, sense), and 5′-TTCTGTATGTCATTGACAGTCCAGCT-3′ (inner, antisense). Both primary and secondary PCR reactions contained 2 units of Taq DNA polymerase (Promega, Madison, WI), 1× PCR buffer (Promega: 1 mM Tris–HCl, 5 mM KCl, 0.1% Triton X-100), 2.5 mM MgCl$_2$, 200 μM of each dNTP, and 0.4 μM of each primer in a total volume of 50 μl. Thermocycling conditions were as follows: 95°C for 2 min and then 35 cycles of 94°C for 30 s, 55°C for

30 s, and 72°C for 1.5 min, followed by a final extension step at 72° for 10 min. PCR products were resolved using 1% agarose gels containing 0.5 μg ethidium bromide and visualized under UV light.

Cloning of PCR products

PCR-amplified DNA was ligated into pGEM-T Easy vectors (Promega), transformed into competent cells (JM 109; Promega), and plated on Luria–Bertani (LB) plates (50 μg ampicillin/ml). Single white colonies were re-plated on fresh LB plates and incubated overnight at 37°C. Colonies were screened by PCR (same primers/conditions as above) and sequencing carried out on mini-prep-extracted DNA.

Sequence analyses

Dideoxynucleotide sequencing reactions were carried out using an ABI Prism Big-Dye Terminator Cycle Sequencing Ready Reaction kit V3.0 (Applied Biosystems, Scoresby, Vic, Australia) as per manufacturer's instructions and sequenced on an ABI 3100 DNA sequencer (Applied Biosystems). Drug resistance profiles were generated using the Stanford HIV database (http://hivdb. stanford.edu/). Sequences were aligned with CLUSTALW (Higgins et al., 1992; Thompson et al., 1994) available through ANGIS (http://www.angis.org.au). Phylogenetic analyses were performed using programs from the PHYLIP package (Felsenstein, 1989) also available through ANGIS.

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