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Polymorphisms of cytotoxic T-lymphocyte (CTL) and T-helper epitopes within reverse transcriptase (RT) of HIV-1 subtype C from Ethiopia and Botswana following selection of antiretroviral drug resistance☆

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Abstract

Drug resistance is the major limiting factor in the effective therapeutic management of HIV infection with antiretroviral drugs (ARVs). In developing countries, where access to ARVs may be limited, therapeutic vaccine protocols designed to restrict the advent of drug resistance may be of interest. Whereas the immunodominant regions of HIV-1 clade B RT peptides have been well characterized, little is known about potential divergence among RTs of other HIV-1 subtypes. In this study, RT sequence polymorphisms were ascertained in phylogenetically classified subtype C isolates from treatment-naïve Ethiopian ($n=5$) and Botswanian persons ($n=9$). There were clusters of variability in some RT epitopes associated with cytotoxic T lymphocyte (CTL) and helper T cell function within subtype C viruses, although other epitopes remained conserved among subtype C and B viruses. Subtype C mutations associated with drug resistance were identified in vitro, using increasing concentrations of non-nucleoside RT inhibitors (NNRTIs) and nucleoside RT inhibitors (NRTIs). Mutations within immunogenic regions of clade C RT were noted during drug selection of subtype C isolates with nevirapine (S98I, Y181C, V108I and K103N), delavirdine, (A62V, V75E, L100I, K103T, V108I, Y181C), efavirenz (K103E, V106M, V179D, Y188C/H, G190A), lamivudine (M184I, M184V), and zidovudine (K70R), respectively. Further characterization of predicted CTL and T-helper anchor motifs and ARV-induced mutations in HIV-1 non-B subtype RTs is warranted.

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1. Introduction

Highly active antiretroviral therapy (HAART) has contributed to a significant reduction in HIV-1-associated mortality and morbidity over the last decade (Palella et al., 1998). This treatment involves the use of potent and expensive antiretroviral drug (ARV) combination regimens that are modified following the emergence of drug resistance (Schinazi et al., 1996; Wainberg and Cameron, 1998; Wainberg, 1999). Although an effective vaccine is the ultimate goal of HIV/AIDS research, alternative preventive measures and ARV management strategies are required to limit the devastating impacts of HIV infection in developing countries (Nabel, 2001; Richman, 2001).

Over the last decade, cumulative data have strongly indicated that host immune response may exert significant antiviral pressure on HIV-1 infection. Therefore, prophylactic and therapeutic vaccine strategies may address the antigenicity of HIV including drug-resistant variants. Each of the HIV *Env*, *Gag*, *Pol*, *Nef*, *Rev*, *Vif* and *Tat* gene products can be immunogenic (Buseyne et al., 1993; Culmann-Penciolelli et al., 1994; Dupuis et al., 1995; Haas et al., 1998; Rowland-Jones et al., 1998; Brander and Goulder, 2000). However, the high level of RT sequence conservation among different HIV isolates makes RT one of the most common targets for cytotoxic T lymphocyte (CTL) recognition; indeed, 80% of HIV-1 infected individuals have RT-specific CTLs (Haas et al., 1998; Samri et al., 2000). The HIV-1 RT sequences that induce strong CTL responses have been defined (Walker et al., 1998; van der Burg et al., 1995; Haas et al., 1998; Samri et al., 2000). HLA class I and II restricted RT epitopes have been characterized using HIV-1 subtype B viruses.

Immune-based therapeutic measures may provide important and less expensive adjuncts to the management of HIV infections with ARVs (Richman, 2001). Among therapeutic immunization strategies that could be considered is the targeting of those HIV-1 RT immunogenic sequences that harbor predicted drug resistance mutations, before the emergence of actual resistant viruses (Myers et al., 2001; Pantaleo, 1997; Richman, 2001). The targeting of CTL responses to drug-resistant variants may improve the long-term efficacy of currently used ARVs by significantly delaying the onset of drug resistance variants.

This vaccine approach is predicted on the notion that ARV therapy selects for viral variants harboring resistance mutations that may alter cellular immune responses (Samri et al., 2000). This has been observed for viruses harboring resistance to lamivudine (3TC), where the presence of the M184V mutation has been associated with the loss of CTL responses to the YMDD motif, highly conserved among retroviruses (Harrer et al., 1996; Schmitt et al., 2000).

Although overall similarities exist among HIV-1 subtypes, there is a need to investigate potential divergences within immunogenic RT sequences among different HIV-1 clades. Epidemics involving HIV group M (non-B, A through J) and O clades are rapidly expanding in the developing world with 50% of new HIV-1 infections in heavily-infected regions of southern Africa and Asia attributable to clade C strains (Dietrich et al., 1993; Burke and McCutchan, 1997; Essex, 1999; UNAIDS, 2000). In as far as treatment in these regions will include less costly drug regimens involving fewer ARVs, the emergence of drug resistance may be more rapid and problematic than that observed for clade B infections in developed nations. It is, therefore, of interest to explore whether conserved RT epitopes exist that could be studied as candidate therapeutic vaccines to delay the onset of resistance.

This study was undertaken to characterize the diversity of RT immunodominant epitopes of HIV-1 subtype C isolates and to determine the potential effects of ARV-induced resistance mutations on recognition of these epitopes by cellular immune responses. A total of 14 HIV-1 clade C treatment-naïve isolates were studied, comprising samples collected from five patients from Ethiopia

and nine from Botswana. Our results demonstrate significant inter- and intra-clade C natural polymorphisms in many epitopes within the RT polymerase domain, although a portion of RT immunogenic sequences appear to be relatively conserved between clades B and C. Additional mutations within the CTL and T-helper epitopes were selected in vitro, using increasing concentrations of different non-nucleoside RT inhibitors (NNRTIs), including nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV), as well as nucleoside RT inhibitors (NRTIs), including lamivudine (3TC) and zidovudine (ZDV).

2. Materials and methods

2.1. Subjects and HIV-1 viral isolation

Blood samples obtained from 14 HIV-1 antiviral treatment-naïve infected African patients were included in the study. Five individuals were from Ethiopia (north-eastern Africa) diagnosed in 1994–1995, shortly after emigrating to Israel. Nine other HIV-1-infected patients were from Botswana (southern Africa). HIV-1 viruses were isolated from Ethiopian and Botswanian peripheral blood mononuclear cells (PBMCs) following co-culture with cord blood mononuclear cells (CBMCs) as previously described (Salomon et al., 1994). Prior to the co-culture, donor CBMCs were stimulated with interleukin-2 (IL-2) (Boehringer-Mannheim, Inc., Montreal, Canada) and phytohemagglutinin (PHA) for 3 days in RPMI-1640 tissue culture medium, as described previously (Salomon et al., 1994). The co-culture medium was supplemented with 10% fetal calf serum, 2 mM glutamine, 200 U/ml penicillin and 200 μM/ml streptomycin, and the cells were incubated at 37 °C in the presence of 5% CO_2. Production of HIV-1 p24 antigen (Abbott Laboratories, North Chicago, IL) and RT activity in cell supernatants were monitored at least once weekly as described (Boulerice et al., 1990; Salomon et al., 1994). Following peak HIV production, amplified viral stocks were isolated and stored at −70 °C until use. The Pol regions of isolates were sequenced as described below. GenBank accession numbers for isolates from Ethiopia were designated 4742 (AF492595) 4743 (AF492596), 4761 (AF492597), 4762 (AF492598) and 4766 (AF492599), whereas the HIV-1 strains from Botswana were BG05 (AF492600), BG15 (AF492601), HSTmok (AF496203), Mol01 (AF492603), Mol03 (AF492604), Mol13 (AF492605), Mol14 (AF492606), Mol18 (AF492607) and Mol36 (AF492608), respectively.

2.2. Subtype assessment by hetero-duplex mobility assays (HMA)

Subtype determination of the different clinical isolates was performed by HMA of *env* regions. For this purpose, reagents and assay protocols were obtained from the AIDS Research and Reference Reagent Program of the NIH (Division of AIDS, NIAID, NIH, MD) (Delwart et al., 1993). Briefly, the C2-V5 *env* region of approximately 0.7 kb of each viral isolate was amplified by a two-round nested PCR amplification reaction, using γ-^{32}P labeled 3′primer. In parallel, other reactions were performed to amplify similar fragments from plasmids harboring *env* genes of a panel of HIV-1 reference strains, representing different HIV-1 subtypes originating from various parts of the world. Equal amounts (5-μl) of PCR products of Ethiopian or Botswanian isolates were mixed with each reference strain in sample tubes containing 1.1 μl of HMA annealing buffer (100 mM NaCl, 10 mM Tris, pH 7.2, 2 mM EDTA). Homo-and hetero-duplex DNA fragments were generated by denaturation at 94 °C for 2 min, reannealed at 4 °C, separated by electrophoresis in 5% acrylamide gels under non-denaturing conditions at 150 V for 4 h, and then visualized by autoradiography.

2.3. Phylogenetic analyses of RT sequences

The subtype status of isolates was confirmed by analysis of RT and protease gene sequences. Viral *Pol* regions, amplified by PCR, were sequenced using the automated TruGene DNA sequencing system (Visible Genetics Inc., Atlanta, GA), as previously reported (Durant et al., 1999). RT sequences of Ethiopian and Botswanian isolates

were aligned and compared with corresponding RT regions of various reference strains representing different HIV-1 subtypes, obtained from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov). Reference subtype strains (with their GenBank accession number and country of origin) included subtype C isolates ETH2220 (U15161 from Ethiopia), 92BR025 (AF009378 from Brazil), IN21068 (AF067155 from India), and 96BW0502 (AF110367 from Botswana); subtype A strains U455 and 92UG0371 (M62320 and AF009398 from Uganda and Kenya, respectively); the subtype D strain NDK (M27323 from Zaire/Congo); the subtype E CM240 and 93TH253.3 isolates (U54771 and U51189 from Thailand) and 90CF402.1 from Central African Republic; the subtype F isolate 93BR020.1 (AF005494 from Brazil); the subtype G SE61165 (AF061642 from Sweden and Zaire); the subtype H 90CF056 (AF005496 from Central African Republic); the subtype J SE92809 (AF082394 from Sweden); the subtype O MVP5180 (L20571 from Cameroon); the subtype CPZ CPZGAB2 (U11495 from Gabon); and the subtype B reference strains LAV and JRFL (04321 and U63632 from France and USA, respectively). Overall, 34 RT nucleotide sequences were analyzed using *GENETOOL* and *PEPTOOL* software (from BIOTOOLS Incorporated, Edmonton, Canada). Each sequence was 397 base pairs in length and the alignments were gap-stripped. The percent identity among selected pairs of RT sequences was determined. The phylogenetic neighbor-joining tree was generated establishing distances between sequences using the phylogenetic computerized programs Dnadist, Neighbor and Drawtree/Drawgram. The parameters used in the tree model was a transmission/transversion ratio of 1.6 and a computer-optimized bootstrap (http://hiv-web.lanl.gov).

2.4. Selection of drug-resistant viral mutants

Subtype C viral variants resistant to the NNRTIs (NVP, DLV and EFV), as well as to the NRTIs (3TC and ZDV), were selected by growing infected CBMCs in the presence of increasing concentrations of these drugs as previously described (Gao et al., 1992). These HIV-1 isolates were repeatedly passaged in CBMCs over 8–30 weeks for NNRTI selections, 8–12 weeks for 3TC, and up to 11 months for ZDV. Selections were initiated with sub-optimal drug concentrations of 0.01 μM for NVP, DLV, and 3TC and 0.001 μM for EFV and ZDV. The final ARV concentrations ranged from 1–10 μM. RT assays were performed weekly to monitor viral replication. When RT activity resurged in association with emergence of drug resistance, genotyping was performed to identify drug-induced mutations. All amino acid substitutions that appeared within the RT CTL and T-helper epitope sequences of the Ethiopian and Botswanian clinical isolates were screened as described below.

2.5. Epitope prediction for HIV-1 clade C RT

The RT protein sequences of the 14 HIV-1 antiviral drug-naïve isolates from Ethiopia and Botswana were analyzed and screened for potential CTL or T-helper restricted anchor residue motifs within RT, using the MultiMotifScanner epitope recognition program at http://hiv-web.lanl.gov that predicts HIV-1 best-suitable sequences for binding to CTL and CD4+T-helper cells (6). Predicted epitope sequences that were conserved and/or varied among HIV-1 clade C strains from Ethiopia and Botswana were analyzed.

3. Results

3.1. Subtype characterization of env genes

Phylogenetic analysis of *env* regions of each of our Ethiopian and Botswanian isolates was determined by HMA. The designation of the HIV-1 subtype of each isolate was based on the relative mobility of duplexes formed with different subtype reference strains. More rapid migration on acrylamide gels of hetero-duplex DNA indicates a relative degree of similarity between the unknown isolate and the reference strain sequence. For comparative purposes, a wild-type (WT) clade G strain (5770) from Gabon was included.

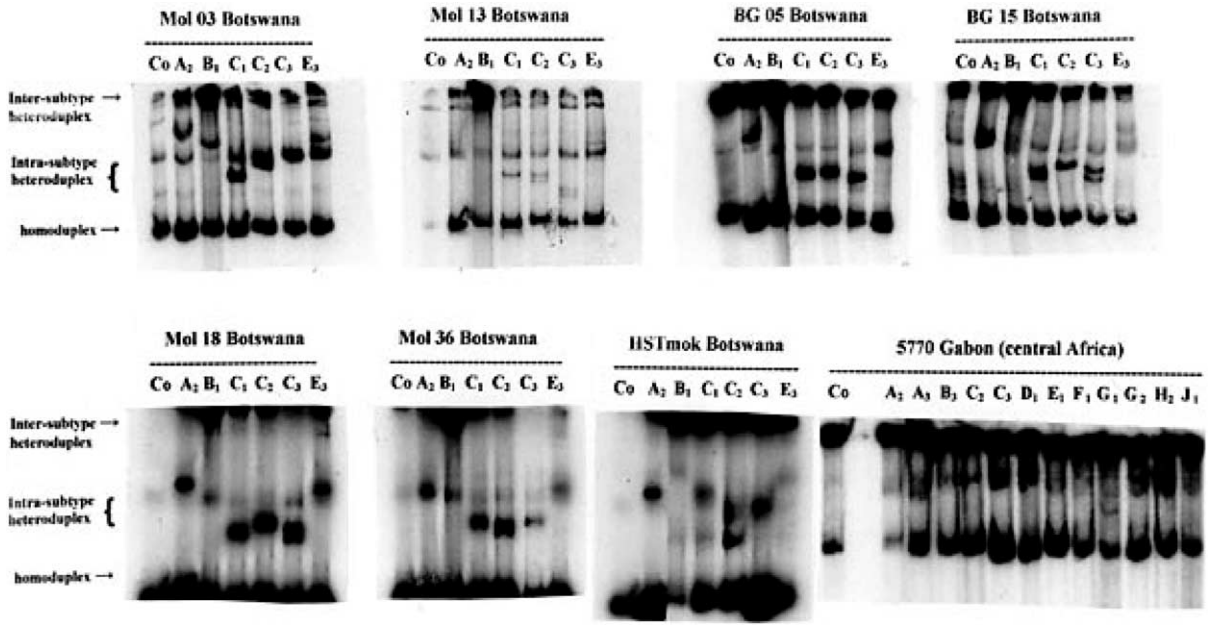

Fig. 1. Heteroduplex mobility analysis of isolates from Botswana drug-naïve patients. Heteromobility duplex assays were performed as described in Section 2. Hetero-complexes were formed by mixing PCR-amplified C2-V5 *env* sequences of subtype reference strains and C2-V5 *env* fragments of Botswanian isolates. A more rapid migration on acrylamide gels of hetero-duplex DNA indicates relative similarity between the unknown isolate and the reference strain. For comparative purposes, a wild-type (WT) HIV-1 clade G strain (5770) from Gabon was used.

The *env* regions of the nine samples from Botswana all belonged to subtype C, as demonstrated by more rapid migration of hetero-duplexes formed with clade C reference strains. Fig. 1 shows an overall view of the HMA profiles of the seven Botswanian samples. The clinical isolate 5770 from Gabon was determined to be a subtype G strain. The subtype C status of the five Ethiopian isolates has been described previously (Loemba et al., 2002).

The set of reference strains used in the HMA reactions was defined as follows: the clade C-C1, C2, C3 reference strains were MA959, ZM18 and IN868 (accession number U08453, L22954, and U07103), respectively; the clade A2, A3 and E1 reference strains IC144, SF170 and TH22 (accession numbers unknown, M66533, and U09131), respectively; the clade B1 and B3 strains BR20 and SF162 (U08797 and M65024, respectively). The samples D1, F1, G1, G2, H2 and J1 designated the strains UG21 (U08804), BZ162 (L22084), RU131 (U08364), LBV21-7 (U09664), and V1557 (U09666), respectively.

3.2. Phylogenetic analysis of RT sequences

A segment encompassing 250 amino acids within the RT molecule was sequenced using automated TruGene DNA sequencing technology. RT nucleotide sequences were aligned with corresponding regions of a set of various reference strains representing different HIV-1 subtypes from diverse regions of the world. RT regions of Ethiopian and Botswanian isolates shared 94.2–97.5% sequence identity at the nucleotide level with each of the four different clade C reference strains, between 90 and 92.5% nucleotide identity with three subtype B reference strains, and between 88.9 and 91.7% nucleotide identity with the subtype A reference strains. The lowest degree of

RT sequence identity of Ethiopian and Botswanian viruses was seen with the clade O reference strain, i.e. 80%.

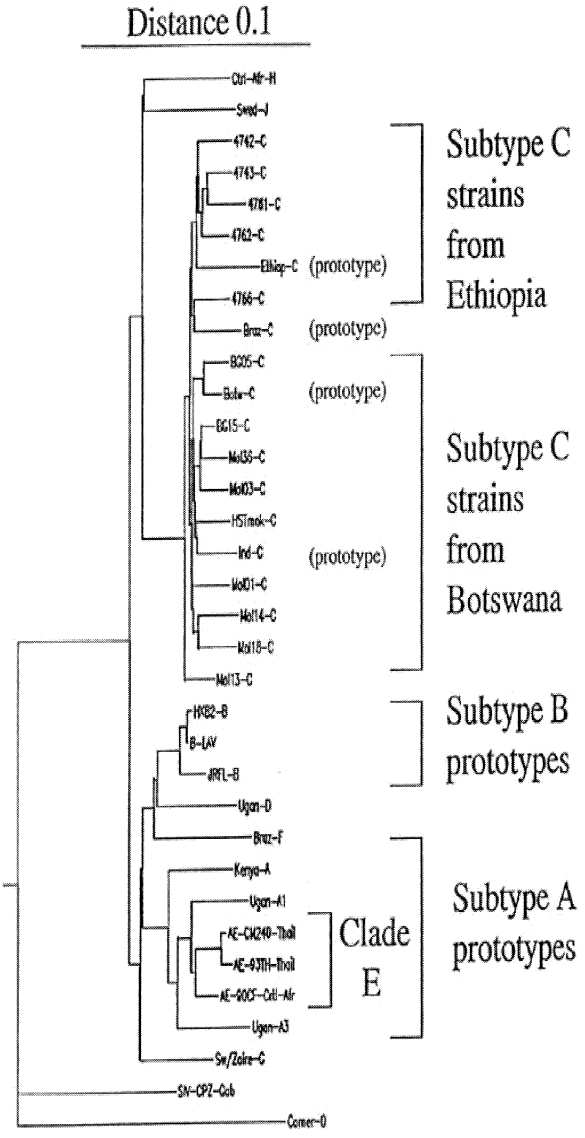

Fig. 2. Phylogenetic analysis of reverse transcriptase sequences of HIV-1 isolates from Ethiopia and Botswana. Phylogenetic analysis comparing the RT regions of HIV-1 *pol* genes from five Ethiopian clinical isolates, nine HIV-1 isolates from Botswana and twenty different prototype strains representing various HIV-1 subtypes. Tree topology was inferred by neighbour-joining method and was based on an alignment of 397 nucleotides from which columns containing gaps were deleted. The subtype O prototype isolate was treated as an outlier.

A phylogenetic tree was constructed by the neighbor-joining method, using the alignment of 34 corresponding RT sequences of the Ethiopian and Botswanian isolates and different HIV-1 subtype prototype strains (Fig. 2). Eleven major branches are seen in the tree, harboring sequences of 11 different subtypes (A, B, C, D, E, F, G, H, J, CPZ, and O). All five isolates from Ethiopia and nine isolates from Botswana clustered with the four different HIV-1 clade C prototype strains from Ethiopia, Brazil, India, and Botswana. Two different sub-clusters were observed in the tree: one representing clade B prototype strains and the other for clade A and the closely related E reference strains.

As observed with HMA assays, phylogenetic characterizaton of RT regions confirmed that all Ethiopian and Botswanian strains were of subtype C.

3.3. *Analysis of genetic variability of RT, CTL, and T-helper epitope sequences*

A set of known HIV-1 RT antigenic regions, corresponding to RT CTL and T-helper epitopes of subtype B, was obtained from the Los Alamos National Library Molecular Immunology Database. The epitope amino acid sequences characterized for the subtype B RT domains were aligned with the corresponding RT sequences of clade C isolates from Ethiopia and Botswana. Sequences were screened for potential genotypic polymorphisms that may exist in specific RT CTL and T-helper epitopes of HIV-1 clade C. Tables 1 and 2 illustrate the polymorphisms within the sequences of Ethiopian and Botswanian clade C isolates. Major genotypic differences were observed in many epitope sequences of clade C viruses, corresponding to the characterized CTL epitope regions of clade B RT (Table 1). A clustering of sequence divergence was also detected within the RT of Ethiopian clade C isolates and the RT regions of Botswana clade C isolates. However, a few RT CTL epitopes were conserved between the subtype B and subtype C strains from Ethiopia and Botswana (CTL epitopes 103–118, 108–123, 118–128, 128–136, 180–190 and 192–201) (Table 1).

Table 1
Sequence divergence within RT cytotoxic T cell epitopes

HIV-1 RT CTL epitopes (Clade B RT domain)		Corresponding polymorphisms in subtype C RT CTL epitopes		Mutations within the clade C RT CTL after selection of drug resistant isolates				
Location	Amino acid sequence	Ethiopian isolates ($n = 5$)	Botswana isolates ($n = 9$)	NVP ($n = 8$)	DLV ($n = 7$)	EFV ($n = 6$)	3TC ($n = 7$)	AZT ($n = 1$)
33–43	ALVEICTEMEK	V35T (4), E36A (3), T39E (5), E40D (1), E40K (1), K43N (1), K43R (1)	T39E (9)	1/7				
42–53	EKEGKISKIGPE	K43N (1), K43R (1), I47F (1), S48T (2)	S48T (8/9), S48E (1/9)					
103–118	KKSVTVLDVGD-AYFSV	–	K104R(1)	K103N (1/8)	K103T (1/7), V108I (1/7)	K103E (1/6), V106M (5/6)		
108–123	VLDVGDAYFSVP-LDED	D123S (3), D123G (2)	D123S (2), D123G (3), D123N (1)	V108I (1/8)	V108I (1/7)			
118–128	VPLDEDFRKYT	D123S (3), D!23G (2)	D123N (2), D123G (3), D123N (1)					
128–136	TAFTIPSIN	I135T (1)	I135T (1)					
157–179	PAIFQSSMTKI-LEPFRKQNPDIV	A158S (2), Q174K (3), K173A (5), D177E (4), D177G (1)	S162A (1), S162C (1), T165I (3), K166R (2), E169K (1), K173T (1), K173E (1), K173A (7), Q174R (1), Q174K (2), D177E (8), I178M (2)			V179D (1/6)		
175–184	NPDIVIYQYM	D177E (4), D177G (1)	D177E (8), I178M (2)	Y181C (5/8)	Y181C (2)	V179D (1/6)	M184I (3/7), M184V (5/7)	
179–190	VIYQYMD-DL(YVG)	G190A (1)	–	Y181C (5/8)	Y181C (2)	Y188C/H (3/6)	M184I (3/7), M184V (5/7)	
192–201	DLEIGQHRTK	T200A (4)	T200A (9)	Y188C (1/8)				
201–210	KIEELRQHLL	Q207E (4), Q207D (1)	I202V (1), E204K (1), Q207E (7), Q207K (1), Q207R (1)					
209–220	LLRWGLTTPDKK	R211K (1), L214F (5)	R211K (6), R211Q (1), L214F (9)					

Amino acid sequences of the known CTL epitopes in subtype B RT were analyzed for natural divergence P236L3/7 in corresponding sequences of clade C RT of Ethiopian and Botswanian isolates. Resistance mutations were selected using increasing concentrations of designated antiviral drugs in tissue culture. The frequency of each mutation or polymorphisms is noted in parentheses for the designated number of isolates indicated in each heading.

Table 2
Sequence divergence within RT T-helper cell epitopes

HIV-1 RT T-Helper epitopes (Clade B polymerase domain)		Polymorphism of corresponding HIV-1 subtype C RT T-Helper epitope sequences		Additional mutations generated within the putative clade C RT T-Helper epitopes after in vitro selection of drug resistant isolates				
Location	Amino acid sequence	Ethiopian isolates ($n = 5$)	Botswanian isolates ($n = 9$)	Nev ($n = 8$)	Del ($n = 7$)	Efv ($n = 5$)	3TC ($n = 7$)	AZT ($n = 1$)
36–53	EICTEMEKEGKISKIGPE	E36A (3), T39E (5), E40D (1), E40K (1), K43N (1), K43R(1)	T39E (9), S48T (8), S48E (1)				T39K (1)	
39–54	TEMEKEGKISKIGPEN	T39E (5), E40D (1), E40K (1) K43N (1), K43R(1), I47F (1), S48T (2)	T39E (9), S48T (8),S48E (1)					
48–73	SKIGPENPYNTPVFAI	S48T (2), N57K(1), D67G (1), T69P (1), K70R (1)	S48T (8), S48E (1)		A62V (1)			K70R (1)
62–78	AIKKKDSTKWRKLVDFR	D67G (1), T69P (1)	–		V75E (1)			
88–100	WEVQLGIPHPAGL	A98S (2)	–	S98I (1)	L100I (1)			
133–148	PSINNETPGIRYQYNV	I135T (1), E138A (1), T139A (1)	I135T (1), E138A (1), I142V (1)					
144–159	YQYNVLPQGWKGSPAI	A158S (2)	A158S (1)					
171–191	FRKQNPDIVIYQYMDDLYVGS	K173A (5), Q174K (3), D177E (4), D177G (1), G190A (1)	K173T (1), K173E (1), K173A (7), Q174R (1), Q174K (2), D177E (8) I178M (2)	Y181C (5)		V179D (1), Y188C/H (3), G190A (1)	M184V (5), M184I (3)	
195–210	IGQHRTKIEELRQHLL	I195L (1), G196R (1), T200A (4), K201N (1), Q207E (4), Q207D (1)	T200A (9), I202V (1), E204K (1), Q207E (7), Q207K (1), Q207R (1)					
196–216	GQHRTKIEELRQHLLRWGLT	G196R (1), T200A (4), K201N (1), Q207E (4), Q207D (1), R211K (1), L214F (5)	T200A (9), I202V (1), E204K (1), Q207E (7), Q207K (1), Q207R (1), R211K (6), R211Q (1), L214F (9)					

Amino acid sequences of the known T-Helper epitopes in subtype B RT were analyzed for natural divergence in corresponding sequences of clade C RT of Ethiopian and Botswanian isolates. Resistance mutations were generated in clade C RT under drug pressure in tissue culture. The frequency of each mutation or polymorphisms is noted in parentheses for the designated number of isolates indicated in each heading.

The RT enzyme has been subdivided into several regions based on its crystal structure including fingers, thumb, and palm domains (Kohlstaedt et al., 1992). The polymorphisms in T helper recognition motifs, existing within RT regions of clade C isolates from Ethiopia and Botswana, was concentrated mainly in the N-terminus portion of the RT fingers sub-domain (CD4 T-cell epitopes 36–53, 39–53 and 48–53) and in the C-terminus portion of the RT palm sub-domain (T-helper epitopes 171–191, 195–210 and 196–216) (Table 2). The C-terminus of the RT fingers region and the N-terminus portion of the palm sub-domain of HIV-1 RT appear to be relatively conserved between HIV-1 clade B and the Ethiopian and Botswanian clade C strains (CD4 T-cell epitopes 62–78, 88–100, 133–148 and 144–159).

3.4. Selection of HIV-1 clade C mutants with ARV in cell culture

In order to predict resistance mutations that may appear within RT epitope sequences, Ethiopian and Botswanian strains were grown in the presence of increasing concentrations of NNRTIs (NVP, DLV, and EFV) and NRTIs (3TC and ZDV). Tables 1 and 2 reveal specific-drug resistance mutations that were generated within clade C RT immunogenic regions. The mutations S98I, Y181C, V108I and K103N were generated during selection with NVP. Incubation with DLV led to previously characterized mutations at sites associated with resistance to DLV (K103T, Y181C) and other NNRTIs (L100I, V108I) and multi-drug secondary resistance to NRTIs (A62V, V75E). Selection with EFV led to the EFV-related mutations V106M, K103E, V179D, Y188C, Y188H, and G190A.

There appears to be an association between the presence of natural polymorphisms in clade C isolates and development of drug escape mutations. This is particularly noteworthy for EFV selections where the observed polymorphism at the valine codon 106, i.e. GTA (clade B) versus GTG (clade C), led to a hitherto unreported V106M mutation (GTG→ATG) in 5 of 6 isolates. In the remaining EFV selection, the valine codon 106, i.e. GTT (clade B) versus GTC (clade C), must have facilitated the V179D mutation (GTC→GTA) conferring EFV resistance.

In contrast, the V106M mutation never arose in NVP selections with eight separate isolates. This is surprising, since the V106A mutation (GTA→GCA) has been described in NVP-treated individuals infected with clade B virus. Moreover, V106A has been associated with NVP resistance and EFV and DLV sensitivity (Wainberg, 1999). However, NFV yielded the Y181C and Y188C mutations in 5 of 8 and 1 of 8 selections, respectively. The natural G190A mutation present in one wild-type isolate conferred innate NVP resistance. In one isolate, NVP and DLV resistance did not develop where the natural V108V polymorphism GTA (clade B) versus GTG (clade C) was present in addition to the V106V and V179V polymorphisms.

With regard to DLV selections, the DLV-specific P236L and K103T resistance mutations were observed in 3 of 7 and 1 of 7 selections, respectively. The L100I and V108I resistance mutations conferring NVP/DLV/EFV cross-resistance arose in the other DLV selections. These DLV resistance patterns were similar to those observed for clade B DLV selections.

Cell culture selection with 3TC generated the mutations M184I and M184V with 3 and 5 of seven isolates, respectively. Interestingly, M184I appeared to be a more stable mutation for clade C than that observed with clade B isolates (unpublished results).

The K70K natural polymorphism (AAA→AAG), present in clade C isolates, led to the K70R mutation in only 1 of 5 ZDV selections. Interestingly, phenotypic resistance to ZDV arose in the remaining four selections, although no RT nucleotide changes were observed between codons 1–250. Clearly, characterization of other regions of Gag-pol is warranted to determine the origin of this ZDV resistance.

Other amino acid substitutions, outside of known drug-resistance sites, were observed in HIV-1 clade C RT regions overlapping CTL and T-helper epitope sequences. These in vitro mutations were rarely observed with single isolates and may not reflect in vivo drug selection. These

include a D186N mutation arising during one NVP selection, T39K in one 3TC selection, and both A173V and I135M with a single ZDV selection. One of seven DLV selections generated V90I, K154E, and F171L, I178M within CD4 T-cell epitopes and D113I, I132L, and F171L, A173V, I178M within CTL epitope regions.

3.5. Clade C RT sequence screening for potential immunogenic motifs

The most conserved RT epitope sequences among all Ethiopian isolates and those conserved among all nine isolates from Botswana were analyzed (Table 3). In the case of Ethiopian strains, complete sequence identity was observed with the Ethiopian HIV-1 clade C reference strain ETH2220 in 14 of the predicted RT epitope sequences. Similarly, Botswanian viruses shared 18 highly conserved sequences with the Botswanian HIV-1 clade C reference strain 96BW0502. Among all predicted immunogenic regions of RT, just three areas, located in the N-terminal portion of the fingers (amino acid 145–154, 146–154 and 147–154), were completely conserved among all clade C strains from Ethiopia and Botswana (Table 3).

4. Discussion

In this study, we have described natural variations and drug-selected mutations in RT immunogenic regions of 14 HIV-1 clade C Ethiopian and Botswanian isolates. These RT regions correspond to known specific CTL and CD4 T-cell anchor sequences in HIV-1 clade B RT. The existence of polymorphisms in the CTL and T-helper epitopes may impact on the immunological recognition of HIV-1 RT regions and immunological cross-reactivity. We have mainly investigated the fingers and palm sub-domains of RT, as the majority of drug resistance mutations are generated within these regions. The phylogenetic characterization of the *env* gene of Ethiopian and Botswana isolates by HMA and phylogenetic analysis of their RT sequences have confirmed that they are related to HIV-1 subtype C strains.

The strong CTL immunogenic properties of HIV-1 RT have been documented by numerous studies (Walker et al., 1998; van der Burg et al., 1995; Haas et al., 1998; Samri et al., 2000; Day et al., 2001). Since *Pol* is among the most conserved genes within different HIV-1 subtypes, RT represents a potential vaccine target for induction of immune cross-recognition among different HIV-1 subtypes. In our study, an average sequence homology of about 90–92.5% was found between Ethiopian and Botswanian clade C isolates and the other HIV-1 group M prototype strains. Despite relatively little sequence variation, large immunological differences may exist among RTs from different HIV-1 subtypes. The various HIV-1 subtype natural polymorphisms that exist at amino acid anchor positions may alter antigen processing and presentation, allowing immune escape of HIV-1 subtype variants (Del Val et al., 1991; Ossendorp et al., 1996; Goulder et al., 1997).

At early stages of HIV-1 infection, the immune system, particularly CD8 cell-mediated CTL function, may help to suppress viral replication (Koup et al., 1994; McMichael and Rowland-Jones, 2001). Strong CTL activity directed against multiple conserved HIV-1 epitopes may be protective against HIV-1 infection (Rowland-Jones et al., 1998; McMichael and Rowland-Jones, 2001). However, selection of variants harboring new mutations in CTL epitopes may enable HIV-1 to evade the cellular immune response. Knowledge of such mutations in RT and elsewhere, including CTL epitopes, among different HIV clades is important.

Clustering of genotypic divergence has also been reported in Gag-specific CTL epitopes (Buseyne et al., 1993), and may be involved in HIV-1 escape from HLA-B27-restricted CTL responses (Kelleher et al., 2001). To date, there has been no report on the genotypic divergence of CTL epitope sequences in clade C RT from different regions.

The importance of T-helper lymphocytes (CD4+cells) in induction of CTL responses is also becoming clearer, although the helper role in maturation of CD8+T-cell functions is suggested but not yet proven (Ridge et al., 1998; McMichael and Rowland-Jones, 2001). We have shown that Ethiopian and Botswanian isolates have clustered

Table 3
Predicted HLA class 1 and class 2 conserved epitopes within RT of HIV-1 clade C isolates from Ethiopia and Botswana

Conserved epitopes among 100% of clade C isolates from Ethiopia		Conserved epitopes among 100% of clade C isolates from Botswana		Conserved epitopes shared among 100% of all clade C isolates from Ethiopia and Botswana		Conserved epitopes shared among 60 – 80% of all clade C isolates from Ethiopia and Botswana	
Location	Sequence	Location	Sequence	Location	Sequence	Location	Sequence
56–64	YNTPVFAIK	38–46	CEEMEKEGK	145–154	QYNVLPQGWK	38–46	CEEMEKEGK
56–65	YNTPVFAIKK	39–46	EEMEKEGK	146–154	YNVLPQGWK	39–46	EEMEKEGK
57–64	NTPVFAIK	61–70	FAIKKKDSTK	147–154	NVLPQGWK	56–64	YNTPVFAIK
57–65	NTPVFAIKK	62–70	AIKKKDSTK			56–65	YNTPVFAIKK
57–66	NTPVFAIKKK	63–70	IKKKDSTK			57–64	NTPVFAIK
58–65	TPVFAIKK	64–73	KKKDSTKWRK			57–65	NTPVFAIKK
58–66	TPVFAIKKK	65–73	KKDSTKWRK			57–66	NTPVFAIKKK
59–66	PVFAIKKK	92–101	LGIPHPAGLK			58–65	TPVFAIKK
73–82	KLVDFRELNK	93–101	GIPHPAGLK			58–66	TPVFAIKKK
74–82	LVDFRELNK	93–102	GIPHPAGLKK			59–66	PVFAIKKK
75–82	VDFRELNK	94–101	IPHPAGLK			63–70	IKKKDSTK
145–154	QYNVLPQGWK	94–102	IPHPAGLKK			64–73	KKKDSTKWRK
146–154	YNVLPQGWK	94–103	IPHPAGLKKK			66–73	KDSTKWRK
147–154	NVLPQGWK	95–102	PHPAGLKK			75–82	VDFRELNK
		95–103	PHPAGLKKK			145–154	QYNVLPQGWK
		96–103	HPAGLKKK			146–154	YNVLPQGWK
		146–154	YNVLPQGWK			147–154	NVLPQGWK
		147–154	NVLPQGWK				

RT protein sequences of five clade C isolates from Ethiopia and nine from Botswana were scanned for identification of putative HLA anchor residue motifs using the computer-based epitope search program 'MULTIMOTIFSCANNER', at the web site http://phage.lanl.gov/cgibin/EPI_PREDICT/MultiMotifScanner.pl. The non-conserved motifs have been excluded from the table.

polymorphisms within certain CD4+T cell epitopes, mainly the N-terminal part of the RT fingers and the C-terminal region of the RT palm subdomain. T-helper epitope diversity in clade C RT may contribute to cross-reactivity of RT regions.

In vivo CTL recognition of RT epitopes can be affected by drug resistance mutations (Dalod et al., 1998; Wainberg and Cameron, 1998; Gray et al., 1999; Wainberg, 1999; Samri et al., 2000). Indeed, the immune system may be able to distinguish between drug-resistant and wild-type virus, as observed for the M184V mutation associated with resistance to 3TC. Cell culture selection experiments, with NNRTIs and NRTIs, using Ethiopian and Botswanian subtype C strains, has revealed common mutations at sites associated with drug resistance, as well as a number of other amino acid changes. Mutations were mainly noted within two relatively conserved immunogenic regions of RT that overlap with CTL epitopes 103–118, 108–123, 175–184, and 180–190. In the case of T-helper epitopes, half of drug resistance mutations arose in a relatively conserved region 48–73, 62–78, 88–100 and the other half in the highly polymorphic epitopes 171–191, 195–210, and 196–216.

We used an epitope prediction approach, effective for defining optimal MHC ligands and peptide motifs. Interestingly, predicted HLA class I and class II epitopes, conserved among subtype C strains from Ethiopia and Botswana, were located in the regions of RT (amino acids 63–82 and 145–154) that span the C-terminus portion of the RT fingers sub-domain and the N-terminus of the palm region. These regions are relatively conserved among clade B and clade C strains, with regard to previously characterized T-helper epitopes. Given the susceptibility of CD4+ and CD8+T cells to epitope sequence variations, inter- and intra-clade variability may have important impact on vaccine development. CTL activity against various clade B antigens (Gag, RT, *env*, and Nef) in patients with non-subtype B viruses was relatively high in subjects infected with clade A and G and low in an individual infected with with clade C (Cao et al., 1997). Whereas clade B RT antigens yielded only 5% CTL-mediated lysis, the same antigens yielded 17–60% lysis when performed with CTLs from patients infected with clade A and G (Cao et al., 1997).

Antiretroviral therapy during acute stages of HIV-1 infection may be beneficial for regeneration of both CD8+ and CD4+T cells and restoration of immune functions (Richman, 2001; McMichael and Rowland-Jones, 2001). A potential therapeutic strategy may involve immunization that results in specific immune responses against mutated segments of RT of different HIV-1 subtypes. This may prime CTL responses to viruses harboring drug resistance mutations prior to administration of therapy (Harrer et al., 1996; Samri et al., 2000; Schmitt et al., 2000). This may improve the efficacy of current antiretroviral drugs by delaying the emergence of drug-resistant variants.

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