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Cross-Clade Human Immunodeficiency Virus (HIV)-Specific Cytotoxic T-Lymphocyte Responses in HIV-Infected Zambians

MICHAEL R. BETTS,[1] JOHN KROWKA,[2] CARLOS SANTAMARIA,[2] KARIN BALSAMO,[1] FENG GAO,[3] GINA MULUNDU,[4] CHEWE LUO,[5] NICHOLAS N'GANDU,[6] HAYNES SHEPPARD,[2] BEATRICE H. HAHN,[3] SUSAN ALLEN,[7] AND JEFFREY A. FRELINGER[1]*

Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7290[1]; Viral and Rickettsial Disease Laboratory, Division of Communicable Disease Control, California Department of Health Services, Berkeley, California 94704-1011[2]; Department of Medicine[3] and Department of Epidemiology, School of Public Health,[7] University of Alabama at Birmingham, Birmingham, Alabama 35294-0008; and Department of Laboratory Medicine,[4] Department of Pediatrics,[5] and Department of Community Medicine,[6] University Teaching Hospital, Lusaka, Zambia

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We have examined cross-clade HIV-specific cytotoxic T-lymphocyte (CTL) activity in peripheral blood of eight Zambian individuals infected with non-B-clade human immunodeficiency virus type 1 (HIV-1). Heteroduplex mobility assay and partial sequence analysis of *env* and *gag* genes strongly suggests that all the HIV-infected subjects were infected with clade C HIV-1. Six of eight C-clade HIV-infected individuals elicited CTL activity specific for recombinant vaccinia virus-infected autologous targets expressing HIV *gag-pol-env* derived from B-clade HIV-1 (IIIB). Recognition of individual recombinant HIV-1 B-clade vaccinia virus-infected targets expressing *gag*, *pol*, or *env* was variable among the patients tested, indicating that cross-clade CTL activity is not limited to a single HIV protein. These data demonstrate that HIV clade C-infected individuals can mount vigorous HIV clade B-reactive CTL responses.

Globally circulating strains of human immunodeficiency virus type 1 (HIV-1) are known to exhibit extraordinary genetic diversity (1, 4, 7, 12, 24). There are currently eight sequence subtypes or clades (A to J), and one outlier group (O) of HIV-1, which have been defined as distinct phylogenetic lineages in evolutionary trees. The extent of HIV-1 sequence diversity (11, 12, 21, 24), intraclade variability (15, 22), and interclade recombination (1, 7, 22) all potentially affect the efficacy of an HIV-1 vaccine.

In HIV vaccine development, a broadly cross-reactive immune response capable of recognizing multiple HIV clades is desirable. While neutralization of different HIV-1 clades by HIV-infected patient sera is limited (14, 17, 19), some gp160-specific human monoclonal antibodies neutralize multiple clades of HIV-1 (18, 26, 27). Additionally, cross-reactive $CD4^+$ T-cell proliferative responses have been observed in HIV-1 B-clade-infected individuals which recognize HIV-1 gp120 V3 loop peptides from A, B, and D-clade viruses (5, 19).

Recently, broadly reactive cross-clade cytotoxic T-lymphocyte (CTL) activity was observed in four recipients of a recombinant canarypox/HIV-1 MN (clade B) gp160 vaccine (6). HIV gp120 clade sequence variation can also influence target recognition by HIV gp120 peptide-specific CTL clones (29). In this report, we demonstrate that CTLs from HIV C-clade-infected individuals kill autologous targets expressing HIV-1 clade B-derived *gag*, *pol*, and/or *env*.

Study subjects (designated ZC01 to ZC13 [see Table 1]) were recruited from a voluntary HIV testing center at Project San Francisco in Lusaka, Zambia (16). The duration of HIV infection in the HIV-seropositive subjects (ZC01 to ZC08) remains unknown. Antibodies to HIV-1 were detected by the dipstick and Capillus tests as described elsewhere (16) and confirmed with a commercial enzyme-linked immunosorbent assay kit (Organon Teknika, Durham, N.C.). None of the patients met criteria for AIDS diagnosis (2), but some suffered from malaria, tuberculosis, and/or other diseases and reported symptoms such as weakness and/or adenopathy, which may have been related to their HIV infections. All HIV-seronegative donors (ZC09 to ZC13) were female partners in discordant couples at the time of blood collection.

Peripheral blood mononuclear cells (PBMC) were isolated from each of the HIV-infected Zambian study participants, and the sequence subtypes of their infecting viruses were characterized by heteroduplex mobility assay (HMA) (*env*) and sequence analysis (*gag*). Figure 1a shows the *env* HMA profile of an HIV-infected donor in the United States (USB01). The HIV *env* region from USB01 is most closely related to the B3, B1, and B2 clade standards but is divergent from the C1 to C4 clade standards. Figure 1b shows a more extensive analysis of the HIV *env* region from Zambian subject ZC08. The HIV *env* region from subject ZC08 was most closely related to the C2 and C1 standards but is divergent from the standards of all other clades. These results are typical of viral *env* sequences from all the HIV-infected Zambian study subjects and suggest that the subjects were infected with C-clade HIV. To confirm subtype classification in a second genomic region, we amplified and partially sequenced a *gag* fragment (620 bp) from the PBMC DNA of each HIV-infected study participant. Phylogenetic analysis (Fig. 2) indicates that all individuals were infected with subtype C viruses. Concordance in two genomic regions (3) strongly suggests that the study subjects were infected with nonrecombinant subtype C viruses.

We next assessed the ability of CTLs from these HIV-1 C-clade-infected Zambians to recognize targets which express

* Corresponding author. Mailing address: Department of Microbiology and Immunology, 609 Mary Ellen Jones Bldg., CB 7290, University of North Carolina, Chapel Hill, NC 27599-7290. Phone: (919) 966-2605. Fax: (919) 962-8103. E-mail: jfrelin@med.unc.edu.

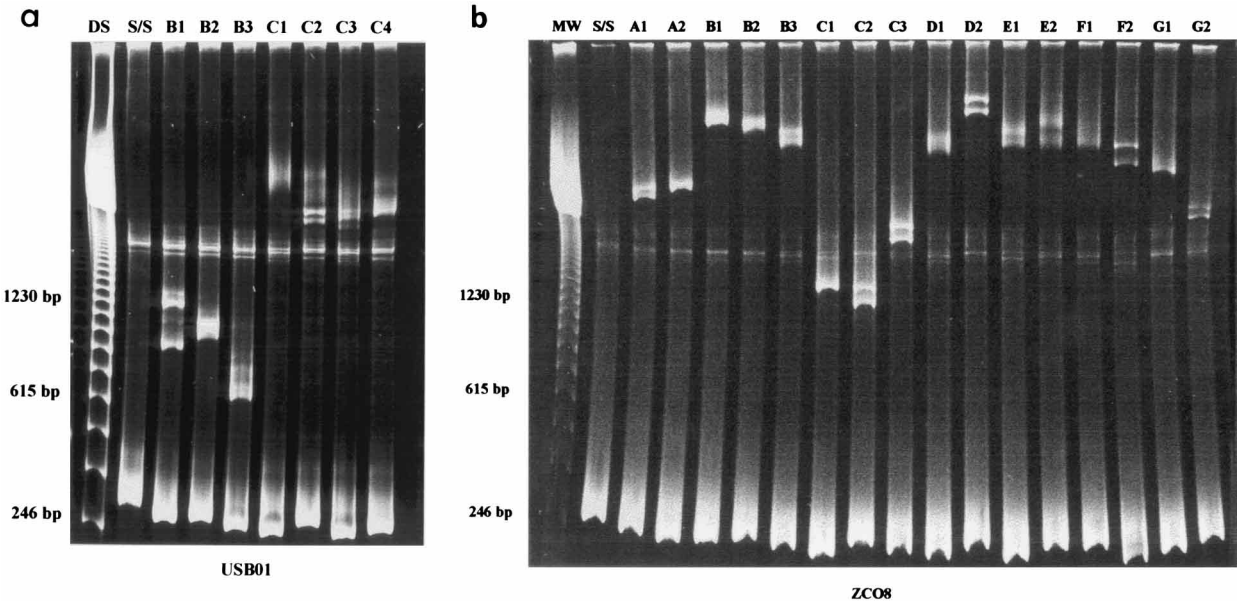

FIG. 1. (a) DNA from an HIV-infected individual in the United States (USB01) was analyzed as described elsewhere (4) by HMA using the ED3, ED14, ES7, and ES8 primers and HIV-1 envelope standards (AIDS Research and Reference Reagent Program) to determine the relatedness of this individual's HIV proviral *env* sequence to clade B and C *env* standards. (b) DNA from an HIV-infected Zambian donor (ZC08) was analyzed by HMA as for panel a to determine the relatedness of the HIV *env* sequence to clade A to G *env* standards. Lanes: MW, molecular weight standards; S/S, PCR products in the absence of clade standards; DS, DNA standards.

HIV-1 B-clade-derived antigens. To selectively restimulate HIV clade B-specific CTL activity, we used UV/psoralen-inactivated, autologous Epstein-Barr virus-transformed B lymphocytes (EBV-BCL) infected with the HIV B-clade vaccinia virus recombinants VV-GPE (VV-ABT 408-6-1, containing clade B HIV-1 IIIB *gag*, *pol*, and *env*; Therion Biologics, Cambridge, Mass.) and VV-nef (containing HIV IIIB *nef*; gift of Andrew McMichael, Oxford, United Kingdom). This technique is highly effective in restimulating major histocompatibility complex (MHC) class I-restricted HIV-specific CTLs from HIV-infected individuals in vitro (9, 13). Freshly thawed donor PBMC were added to the inactivated VV-GPE and VV-nef-infected EBV-BCL at a ratio of 2×10^6 PBMC to 2×10^5 EBV-BCL/well in complete RPMI supplemented with 25 ng of recombinant human interleukin 7 (IL-7) per ml (Endogen, Cambridge, Mass.) and 10% Lymphocult T-LF (IL-2 source; Biotest, Dreiech, Germany) in a 24-well plate (Becton Dickinson, Franklin Lakes, N.J.). The cultures were incubated for 7 days in 5% CO_2 at 37°C and fed at day 3 or 4 by exchanging 1 ml of medium with complete RPMI plus IL-2.

Table 1 shows the CTL responses of eight clade C-infected (ZC01 to ZC08) and five HIV-seronegative (ZC09 to ZC13) donors to HIV-1 B-clade *nef*- and GPE-expressing autologous EBV-BCL. Six of eight C-clade HIV-infected Zambians demonstrated significant (i.e., ≥10% above the level of the vaccinia virus control) HIV-1-specific CTL activity to HIV-1 clade B VV-GPE. Marginal HIV-1-specific CTL activity to VV-nef was detected in ZC02. No HIV-1-specific CTL responses were detectable in the five HIV-seronegative donors.

To further define the specificity of cross-clade CTLs in the clade C HIV-infected subjects, we examined the HIV-specific CTL activity against B-clade HIV-1 gag, pol, and env individually. Autologous EBV-BCL were infected overnight with VV-sc11 or HIV B-clade vaccinia virus recombinants, VV-GPE, VV-gag, VV-pol, or VV-env (VV-gag and VV-pol were from Andrew McMichael; VV-env [vPE16] was from Patricia Earl and Bernard Moss, AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases) at a multiplicity of infection of 5 for use as targets in a standard ^{51}Cr release assay (20). Donor ZC08 (Fig. 3a) demonstrated extensive cross-clade HIV-specific CTL re-

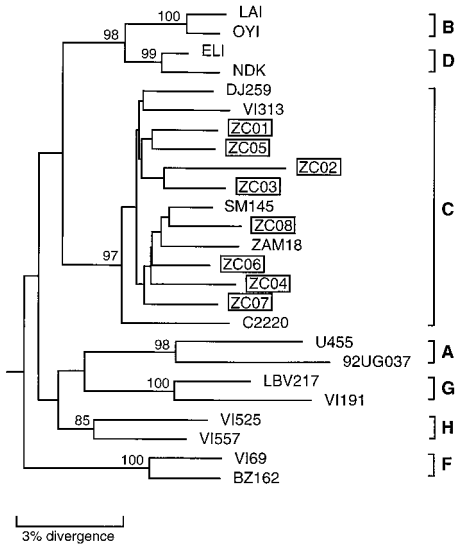

FIG. 2. Phylogenetic relationships of the Zambian viruses (boxed) in comparison to representatives of HIV-1 group M subtypes A to H from the database. Partial *gag* sequences were amplified by nested PCR from uncultured patient PBMC DNA (outer primers: PBS1A, 5′-TTTGCCTGTACTGGGTCTCTCTGGTT-3′, and CgagA, 5′-TGATAAAACCTCCAATTCCCCCTAT-3′; inner primers: PBS1C, 5′-GCTTAAGCCTCAATAAAGCTTGCCTT-3′, and CgagB, 5′-AAT ACTGTATCATCTGCTCCTGTAT-3′) and sequenced either directly or after being subcloned into pCRII by T/A overhang. Nucleotide sequences were aligned by using CLUSTAL (8), with minor manual adjustments. Pairwise evolutionary distances were estimated by Kimura's two-parameter method (10) to correct for superimposed hits. Phylogenetic trees were constructed by the neighbor-joining method (23), and their reliability was estimated from 1,000 bootstrap replicates.

TABLE 1. HIV clade B-specific CTL activity in HIV-infected and HIV-seronegative Zambians

Patient (HIV status[a])	% Specific lysis[b]		
	sc11	*nef*	GPE
ZC01 (+)	0.2	NT	5.1
ZC02 (+)	16.4	24.8	58.5
ZC03 (+)	6.8	NT	13.1
ZC04 (+)	0.0	NT	34.1
ZC05 (+)	6.7	NT	28.3
ZC06 (+)	9.4	NT	62.4
ZC07 (+)	4.3	3.7	31.0
ZC08 (+)	10.1	13.2	62.0
ZC09 (−)	9.0	9.0	9.0
ZC10 (−)	3.0	1.0	4.0
ZC11 (−)	10.0	NT	13.0
ZC12 (−)	11.0	9.0	7.0
ZC13 (−)	14.0	NT	13.0

[a] Determined as described elsewhere (16) and confirmed by enzyme-linked immunosorbent assay.

[b] Determined by ^{51}Cr release assay using autologous EBV-BCL infected with control (sc11) or recombinant vaccinia virus expressing either HIV-1 *nef* or GPE. Results are expressed as means for triplicate wells at an E:T ratio of 50:1 in all subjects except ZC05 (25:1) and ZC01 (20:1), determined as (cpm sample − cpm spontaneous)/(cpm Triton − cpm spontaneous) × 100. Unlabeled sc11-infected EBV-BCL were added to each well at a 20:1 to 40:1 unlabeled-to-labeled cell ratio.

sponses (i.e., greater than 10% above background sc11 specific lysis) reactive with B-clade *gag*-, *pol*-, and *env*-expressing target cells. In ZC08, both *gag*- and *pol*-expressing targets were lysed (20% at a 1:1 effector-to-target [E:T] ratio), and the *env* response was similarly high (20% at an E:T ratio of 5:1). Further analysis of cross-clade CTL reactivity in HIV clade C-infected donors ZC01, ZC05, and ZC06 revealed similar variability of cross-clade recognition to ZC08 (Fig. 3b). Donors ZC08 and ZC06 responded to clade B *gag*-, *pol*-, and *env*-expressing targets, while ZC05 responded only to clade B *gag*-expressing targets. Donor ZC01 failed to recognize clade B GPE-infected targets (Table 1) but demonstrated marginal (10% specific lysis) reactivity to B-clade *pol*-expressing targets. A comparative analysis of the B-clade-specific CTL responses in eight clade B HIV-1-infected subjects and the clade C-infected subjects demonstrated both comparable levels of killing and comparable specificity profiles (data not shown). Taken together, these data demonstrate both the existence and variability of cross-clade reactivity of HIV-specific CTL activity between HIV-1 clades B and C in HIV C-clade-infected Zambians. Whether the clade C-infected donors in this study elicit cross-clade CTL activity against the other known HIV viral clades, i.e., A and D to J, or group O, is currently being studied.

The conservation of CTL epitopes between different HIV-1 clade variants, as well as the host MHC profile, is likely to influence cross-clade CTL recognition. An examination of sequences from the Los Alamos National Laboratory database revealed many conserved CTL epitopes between HIV HxB2r (B clade), Zam18, -19, and -20 (Zambian C-clade isolates), and ETH2220 (Ethiopian C-clade isolate). In HIV-1 gag, 16 CTL epitopes completely conserved between HxB2r and Zam18 to -20 have been identified (9 of 39 known p24 epitopes, 6 of 22 p17 epitopes, and 1 of 2 p15 epitopes). In HIV-1 pol, 9 CTL epitopes completely conserved between HxB2r and ETH2220 (C-clade isolate) are known (0 of 3 protease epitopes, 8 of 31 reverse transcriptase epitopes, and the 1 integrase epitope). In HIV-1 env, however, only 7 CTL epitopes fully conserved between HxB2r and Zam18 to -20 have been described (3 of 41 gp120 epitopes and 4 of 24 gp41 epitopes). This is likely to be an underestimate of functional conservation, since some CTL epitope positions are tolerant of substitution (20, 25). The completely conserved gag, pol, and env CTL epitopes bind a variety of MHC class I alleles, the majority of which are found in south-central Africa (HLA-A2, B7, B8, B14, B35, and B57) (28). The two HIV-infected individuals in this study who failed to elicit cross-clade killing either are poor responders or express different MHC class I haplotypes, which bind CTL epitopes not conserved between HIV-1 B- and C-clade viruses.

Whether cross-clade CTL activity can be primed depends on two important factors: the viral-sequence diversity of the different clades (11, 12, 21, 24) and the MHC class I profile in the infected host or vaccine recipient (6, 29). The generation of an HIV vaccine designed to elicit HIV-specific CTL activity must take these factors into account to prime effective intraclade and interclade CTL activity.

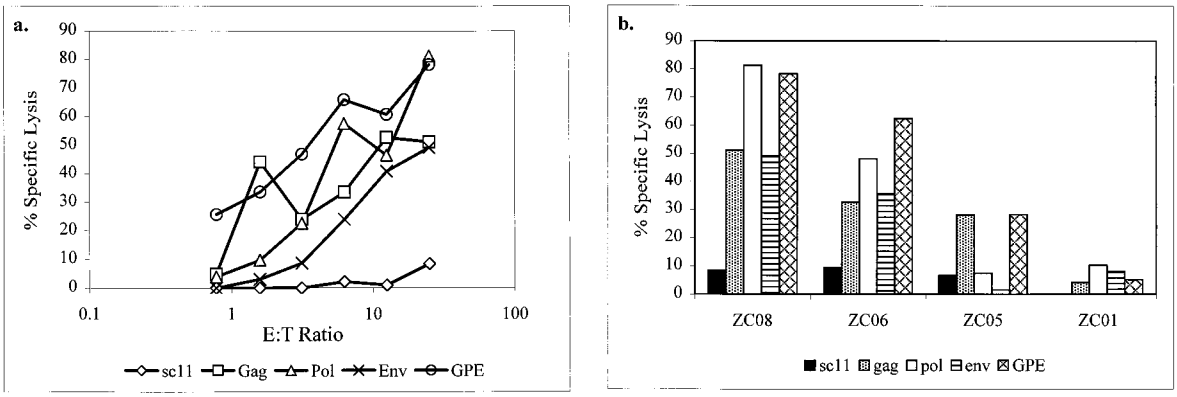

FIG. 3. Zambian subject ZC08 elicits HIV-1 B clade-specific CTL activity against HIV-1 gag, pol, env, and GPE (a), and Zambian subjects ZC08, ZC06, ZC05, and ZC01 elicit variable HIV-1 B clade-specific CTL responses (b). A ^{51}Cr release assay was performed by using uninfected, vaccinia virus control (sc11), and HIV-1 clade B *gag*, *pol*, *env*, or GPE recombinant vaccinia virus-infected EBV-BCL as targets. For panel a, E:T ratios of 25, 12.5, 6.25, 3.1, 1.5, and 0.75:1 were used. For panel b, the E:T ratios were as follows: ZC08, 25:1; ZC06, 25:1; ZC05, 25:1; ZC01, 20:1. Percent specific lysis was calculated as described for Table 1. Unlabeled sc11-infected EBV-BCL were added to each well at a 30:1 unlabeled-to-labeled cell ratio to reduce vaccinia virus-specific background. Lysis of uninfected target cells was below 5% at each E:T ratio tested.

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