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Long-term culture and fine specificity of human cytotoxic T-lymphocyte clones reactive with human immunodeficiency virus type 1

(AIDS/cell-mediated immunity/reverse transcriptase/immunogenic viral epitopes)

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ABSTRACT **The definition of human immunodeficiency virus type 1 (HIV-1) immunogenic epitopes is central to the rational design of AIDS vaccine strategies. In this study, we have generated seven HIV-1 reverse transcriptase-specific cytotoxic T-lymphocyte (CTL) clones from the peripheral blood of two seropositive subjects. Epitopes recognized by these CTL clones were identified by using target cells infected with recombinant HIV-1–vaccinia virus vectors expressing truncated reverse transcriptase proteins and further defined by using target cells incubated with overlapping 25-amino acid synthetic reverse transcriptase peptides. Five different CTL epitopes were identified, and in each case recognition was restricted by class I human leukocyte antigens (HLA). Clones maintained specific cytolytic function in continuous culture for up to 11 months, requiring only periodic restimulation with a CD3-specific monoclonal antibody. These results indicate that HIV-1-specific, major histocompatibility class I-restricted CTL recognize multiple epitopes of a single viral gene product in conjunction with different host HLA antigens. In addition, they demonstrate that human virus-specific CTL can be grown in long-term culture without the need for reexposure to viral antigen.**

Human immunodeficiency virus type 1 (HIV-1) infection is usually associated with a persistent viremia; virus is readily cultured from the peripheral blood of infected individuals (1). Despite ongoing exposure to the virus, most persons remain healthy for years after infection, suggesting that a virus-specific host immune response plays a role in maintaining the asymptomatic state. Cytotoxic T-lymphocytes (CTL) may be an important host defense, as has been demonstrated in other viral infections, including those caused by retroviruses (2–7). CTL responses against the major HIV-1 gene products have been detected in infected individuals (8–15); however, little information is available on actual epitopes recognized by these CTL. An immunodominant T-cell epitope has been identified in mice immunized with a recombinant vaccinia virus expressing the HIV-1 envelope gene product (16). In addition, a human leukocyte antigen (HLA)-B27-restricted *gag* peptide epitope has been identified, using bulk stimulated peripheral blood mononuclear cells (PBMC) from a seropositive individual as effector cells (12). Whereas human CTL clones responsive to HIV-1 have been reported (13), further characterization of epitopes has been hampered, at least in part, by difficulties in propagating human CTL clones *in vitro*.

Here we report a method for generating and maintaining HIV-specific CTL clones in continuous culture. This, in turn, has facilitated a detailed analysis of the fine specificity and major histocompatibility complex (MHC) restriction of these CTL. In these studies we have concentrated on HIV-1 reverse transcriptase (RT)-specific CTL clones, since *pol* is the most highly conserved gene among sequenced viral isolates and RT is a target of both humoral and cellular immune responses in infected individuals (10, 17). Our results indicate that the CTL response to HIV-1 is heterogeneous, in that multiple epitopes of a given viral gene product are recognized in conjunction with different host HLA antigens and that a single HLA antigen can serve as the restricting element for more than one of these epitopes. This work has been presented, in part, elsewhere.¶

MATERIALS AND METHODS

Effector Cells. PBMC were obtained by Ficoll/Hypaque density gradient centrifugation of blood from two asymptomatic HIV-1-seropositive subjects (subjects 63 and 68). These PBMC were either used directly as effector cells in a cytotoxicity assay or cloned at limiting dilution. For cloning, cells were seeded at 50 cells per well in 96-well plates. Irradiated allogeneic PBMC from HIV-1-seronegative subjects were added as feeder cells at 2×10^5 cells per well, in a final volume of 200 μl (per well) of medium [RPMI 1640 medium containing 10% (vol/vol) heat-inactivated fetal calf serum, antibiotics, and 100 units of recombinant interleukin-2 (DuPont) per ml]. The CD3-specific monoclonal antibody 12F6 (8) was added at 0.05–0.1 μg/ml as a stimulus to T-cell proliferation. Plates were incubated at 37°C in a humidified chamber in 5% CO_2 and fed 1 or 2 times per week with medium exchanges. After 3–4 weeks, cells from wells showing cell proliferation were transferred to 24-well plates and restimulated by adding 2 ml of medium containing 2×10^6 irradiated allogeneic feeder cells plus 12F6 at 0.05–0.1 μg/ml. At no time were cells exposed to exogenous viral antigens. Cells were tested 1–3 weeks later for CTL activity. HIV-1 antigen-specific activity was detected in 4% or less of wells showing proliferation, and no cultures with specificity for more than one HIV-1 antigen were detected. These cultures were therefore likely to represent clonal expansion of a single virus-specific cell and, due to their demonstrated unique specificities, are operationally referred to as clones.

Abbreviations: HLA, human leukocyte antigen; MHC, major histocompatibility complex; RT, reverse transcriptase; PBMC, peripheral blood mononuclear cells; EBV, Epstein–Barr virus; CTL, cytotoxic T lymphocytes; HIV-1, human immunodeficiency virus type 1.

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Target Cells. (*i*) *Epstein–Barr virus (EBV) lymphoblasts infected with recombinant HIV-1–vaccinia viruses.* EBV-immortalized B-cell lines were established and maintained as described (8). These were infected with recombinant vaccinia viruses containing the bacterial β-galactosidase gene (VAC/lac, a negative control) or the *env, gag,* or RT genes of HIV-1 (VAC/env, VAC/gag, and VAC/RT, respectively) and were used as target cells in a 4- or 6-hr chromium release assay (8, 10).

(*ii*) *EBV lymphoblasts infected with recombinant vaccinia viruses containing truncated RT gene inserts.* The RT gene of HTLV-IIIB subclone HXB.2 (18), with added start and stop codons, was excised from the replicative form of the M13 bacteriophage recombinant mpCF19 (described in ref. 19) and ligated into the *Sma* I site of pUC19, yielding the recombinant plasmid pCF31. The following six restriction fragments were then purified from this plasmid: *Xba* I–*SSt* I, *Xba* I–*Asp*718, *Xba* I–*Bsp*1286, *Xba* I–*Hin*fI, *Xba* I–*Pvu* II, *Xba* I–*Eco*RV. These were then blunt-ended with the Klenow fragment of DNA polymerase I for ligation into the *Stu* I site of pSC11ss, a vaccinia expression vector derived from pSC11 (20) and containing *Stu* I and *Sal* I sites in place of the *Sma* I cloning site, as well as stop codons in all three reading frames immediately downstream from the *Stu* I site (P. Earl, personal communication). Plasmids containing correctly oriented RT fragments were then used to transfect vaccinia-infected cells, and the resulting recombinant viruses were identified, grown, and purified as described (18). Expression of the truncated RT peptides in cells infected with recombinant virus containing all but the smallest (amino acids 168–315) insert was confirmed by Western blotting (data not shown). Infection of autologous EBV blasts with these vaccinia truncation constructs was performed as described (8, 10), and these cells were then used as targets in a 4-hr chromium release assay.

(*iii*) *EBV lymphoblasts incubated with synthetic HIV-1 RT peptides.* Synthetic HIV-1 RT peptides were synthesized as described (21), using the sequence of the pv22 clone of HIV-1 (22). Peptides overlapped with adjacent peptides by eight amino acids. Chromium-labeled EBV lymphoblasts were incubated for 45 min with the designated peptide at 20 μg/ml, and then autologous RT-specific CTL clones were added as effector cells. The percent specific chromium release was determined after a 4-hr incubation.

RESULTS

Generation of HIV-1-Specific CTL Clones. PBMC were obtained from two asymptomatic HIV-1 seropositive subjects shown to have cytolytic responses to autologous EBV-immortalized B-cell targets expressing the HIV-1 envelope and RT proteins (Table 1). The PBMC were cloned at limiting dilution, a CD3-specific monoclonal antibody was used as a stimulus for T-cell proliferation, and recombinant interleukin 2 was added as a growth factor. Developing clones were tested for virus-specific lysis by using a panel of autologous EBV-immortalized B-cell targets expressing the HIV-1 envelope, *gag,* or RT protein (8–10). After several weeks in culture, 1–4% of developing clones demonstrated HIV-1-specific CTL responses, specific for either the viral RT or envelope (Table 1). No HIV-1 *gag*-specific CTL were detected with either fresh PBMC or cloned cells from these two subjects. At no time were these cells stimulated with viral antigen *in vitro,* indicating that their viral specificities had been determined *in vivo.* Clones demonstrated no evidence of natural killer activity, as assayed by lysis of K562 cells (data not shown).

MHC Restriction of HIV-1 RT-Specific CTL Clones. Clones demonstrating RT-specific cytotoxicity were examined in detail to further define the CTL/target cell interaction. Recognition of virus-infected cells by CTL has been shown to require association of a processed viral protein product with a "self" MHC molecule, either class I (A, B, or C locus) or class II (D locus) (23, 24). To examine the role of the MHC in restricting the cytotoxic activity of each of these clones, allogeneic target cells partially HLA-matched with the donor of the CTL were infected with a recombinant vaccinia virus expressing the full-length HIV-1 RT protein (10, 19). As shown in Tables 2 and 3, for each RT-specific clone a single class I antigen was identified as the restriction element. For subject 63, two clones (63A3 and 63H24) were restricted by

Table 1. HIV-1-specific CTL clones from two seropositive subjects

Subject	Effector cells	E:T ratio	% specific cytotoxicity of target cells: VAC/lac	VAC/env	VAC/gag	VAC/RT
63	PBMC	50:1	5	39	1	25
	63A3	5:1	1	2	1	41
	63A7	5:1	3	0	2	72
	63D35	5:1	1	0	3	48
	63D45	2.5:1	2	72	2	4
	63H24	5:1	1	1	2	56
	63I9	*	1	5	7	44
68	PBMC	50:1	4	34	7	43
	68A13	2.5:1	2	1	4	82
	68A33	25:1	4	29	2	0
	68A62	2.5:1	6	2	4	71

Fresh PBMC and cloned cells were tested for specific lysis of autologous EBV-immortalized B-cell targets expressing the recombinant *env, gag,* or RT protein (VAC/env, VAC/gag, and VAC/RT, respectively) or a control target cell (VAC/lac). For the initial screening, clones were not counted but were used at an effector-to-target (E:T) ratio estimated to be 10:1 (*). All clones except 63I9 were subsequently retested against these four targets at the specific effector-to-target ratios shown. Lysis of autologous target cells by fresh bulk PBMC from each subject is shown for comparison. Average percent spontaneous lysis for the assays shown in this table was always <30%, except for the assay using clone 63A7, where it was 38%.

Table 2. HIV-1 RT-specific CTL clones from subject 63 are MHC class I-restricted

Target cell (shared Ag)	% RT-specific lysis: 63A3	63A7	63D35	63H24	63I9
Autologous	100	100	100	100	100
A1, DQw2	0	0	2	0	NT
A11, DRw52	6	83	111	6	108
A1, A11, DRw52, DQw2	NT	NT	NT	NT	93
B8	149	12	3	NT	NT
A1, B8, DR3, DRw52	91	0	0	75	1
Bw62, DRw52	NT	0	0	1	8
DR3	NT	NT	NT	2	NT
None	NT	NT	NT	1	NT

CTL clones were tested for their ability to lyse allogeneic RT-expressing EBV lymphoblasts matched at specific HLA class I and class II loci, as indicated. Results represent RT-specific lysis expressed as the percentage of that observed against autologous RT-expressing target cells. Clones 63D35 and 63H24 were tested at an effector-to-target ratio of 5:1, and clones 63A3, 63A7, and 63I9 were tested at a ratio of 10:1 in 4-hr chromium release assays. Average percent specific lysis of the autologous RT-expressing target cell and the paired vaccinia control target cell for each clone was 65% vs. 6% for 63A3, 72% vs. 3% for 63A7, 66% vs. 1% for 63D35, 62% vs. 1% for 63H24, and 88% vs. 3% for 63I9. Average spontaneous lysis for these assays was always <30%. NT, not tested; Ag, antigen. The complete HLA type of subject 63 is A1, A11; B8, Bw62; Cw4, −; DR3, −; DQw2; DRw52.

Table 3. HIV-1 RT-specific CTL clones from subject 68 are MHC class I-restricted

Target cell (shared Ag)	% RT-specific lysis	
	68A13	68A62
Autologous	100	100
A2, DR4	7	96
A2, B7	10	82
A2, B7	0	106
A32	12	4
B7, DR1	0	2
Bw60	108	0
None	5	2

Clones were tested as described in Table 2, except that an effector-to-target ratio of 2.5:1 was used in 4-hr chromium release assays. Average percent lysis of the autologous RT-expressing target cell and the paired vaccinia control target cell for each clone was 39% vs. 3% for 68A13 and 59% vs. 2% for 68A62. Average spontaneous lysis for these assays was always <30%. The complete HLA type of subject 68 is A2, A32; B7, Bw60; Cw3, w7; DR1, 4; DQw1, 3; DRw53.

the HLA-B8 antigen, whereas three others (63A7, 63D35, and 63I9) were restricted by the HLA-A11 antigen (Table 2). The two RT-specific clones derived from subject 68 (68A13 and 68A62) were restricted by the HLA class I antigens Bw60 and A2, respectively (Table 3). Phenotypic analyses revealed the clones to be $CD3^+$, $CD8^+$ (data not shown). In no instance was class II-restricted CTL activity (25) detected using the cloning strategy we employed.

CTL Lysis of Target Cells Expressing Truncated RT Gene Products. The viral epitopes recognized by these RT-specific clones were then investigated with a series of recombinant vaccinia viruses constructed to express the RT protein with sequentially greater carboxyl-terminal deletions, as shown in Fig. 1. Six different vectors were used, expressing from 100% (amino acids 168–706) to 27% (amino acids 168–315) of the RT protein (18). Clones 63A3 and 63H24, both restricted by the HLA-B8 antigen, were able to lyse target cells infected with each of the truncated vectors, even when only the amino-terminal 148 residues of RT were expressed (amino acids 168–315). These results indicate that the epitope recognized by these clones lies somewhere in this amino-terminal region. Lysis by clones 63A7 and 63D35, both restricted by the HLA-A11 antigen, was completely abrogated when amino acids 316–422 were no longer expressed. In contrast, a third HLA-A11-restricted clone (63I9) from this subject recognized an entirely different epitope, in that CTL activity was lost when amino acids 481–531 were no longer expressed. Clone 68A62 from subject 68 also recognized a viral epitope in this same region (amino acids 481–531), whereas amino acids lying in the region 316–422 were essential for recognition by clone 68A13 from this subject.

CTL Lysis of Target Cells Incubated with Synthetic HIV-1 RT Peptides. HLA class I-restricted CTL generally recognize viral antigen that has been processed endogenously in infected cells and presented at the cell surface as a binary complex with the restricting HLA molecule (26). Target cells can also be sensitized to virus-specific lysis *in vitro* by incubation with the appropriate synthetic viral peptide (27), presumably through formation of a binary complex involving synthetic viral peptide and surface HLA class I molecule of the uninfected target cell. The fine specificity of the RT-specific clones was therefore investigated by using autologous target cells incubated with overlapping 25-amino acid synthetic peptides derived from the predicted amino acid sequence of the RT protein (21). As shown in Table 4, target cells were sensitized to lysis by either a single peptide or two adjacent peptides in the regions predicted by the truncation constructs to contain the CTL epitopes. Clones 63D35 and 63A7 were able to lyse cells incubated with either peptide 49 or 50, suggesting that the epitope recognized lies within the 8-amino acid overlap region (amino acids 342–349). For all other clones, target cells were sensitized by only a single peptide. For clones 68A62 and 63D35, MHC restriction was also investigated with peptide-sensitized target cells and showed the same MHC restriction specificity as had been demonstrated using recombinant vaccinia virus to express the full-length RT protein in autologous cells (data not shown).

Long-Term Culture of HIV-1-Specific CTL. Two of these CTL clones have now maintained virus-specific CTL activity in continuous culture for 10 and 11 months, respectively, requiring only periodic restimulation with the CD3-specific monoclonal antibody. For example, after 315 days in culture, clone 68A62 demonstrated 54% specific lysis of autologous RT-expressing target cells at an effector-to-target ratio of 2.5:1, with only 3% lysis of the paired control target cell (Table 5). Maintenance of virus-specific killing has not required *in vitro* exposure to viral antigen. Analysis of supernatant fluid from the clones for HIV-1 p24 antigen (28) as well as analysis of cloned cells by polymerase chain reaction (29, 30) for evidence of HIV-1 genomic sequences have both been

RT AMINO ACIDS EXPRESSED	% SPECIFIC CYTOTOXICITY						
	63A3 (B8)	63A7 (A11)	63D35 (A11)	63H24 (B8)	63I9 (A11)	68A13 (Bw60)	68A62 (A2)
168-706	75	75	80	69	67	50	72
168-598	72	80	93	82	67	46	74
168-531	73	69	82	79	79	45	89
168-480	75	79	91	72	0	41	0
168-422	88	86	101	62	0	47	1
168-315	70	0	7	78	2	2	1

FIG. 1. Specific lysis of target cells infected with recombinant vaccinia viruses expressing truncated RT gene products. A panel of vaccinia viruses containing sequential carboxyl-terminal truncated HIV-1 RT gene inserts were used to infect EBV-immortalized B cells from subjects 63 and 68. Autologous clones were then tested for their ability to lyse these cells. Results are shown at an effector-to-target ratio of 25:1, except for clone 63I9, which was tested at a ratio of 10:1. Similar results were obtained when clones were used at an effector-to-target ratio of 2.5:1. Average spontaneous lysis for these assays was always <30%. The amino acids expressed by these constructs are indicated; the numbers correspond to the *pol* gene sequence of HIV-1 (17). Amino acids 168–706 represent the full-length RT protein.

Table 4. Lysis by RT-specific CTL clones of autologous lymphoblasts incubated with synthetic 25-amino acid RT peptides

Peptide		% specific cytotoxicity						
no.	Residues	63A3	63A7	63D35	63H24	63I9	68A13	68A62
39	155–179	4	0	0	0	—	—	0
40	172–196	15	0	2	16	0	—	0
41	189–213	8	2	2	0	—	—	0
42	206–230	0	7	9	0	—	—	0
43	223–247	2	0	0	0	—	—	0
44	240–264	1	0	0	0	—	—	0
45	257–281	2	0	0	0	—	0	0
46	274–298	2	0	2	0	—	0	0
47	291–315	3	2	0	0	—	0	0
48	308–332	8	0	0	0	—	1	0
49	325–349	0	89	90	0	1	1	0
50	342–366	0	67	50	0	0	2	0
51	359–383	6	5	2	0	3	14	0
52	376–400	—	0	0	0	2	2	0
53	393–417	1	0	0	0	0	1	0
54	410–434	—	0	3	0	2	0	0
55	427–451	—	—	—	NA	NA	—	NA
56	444–468	—	—	—	0	0	—	0
57	461–485	0	6	3	0	4	0	31
58	478–502	—	—	—	—	0	—	0
59	495–519	—	—	—	—	68	—	0
60	512–536	—	—	—	—	2	—	1
61	529–553	—	—	—	—	2	—	0
62	546–570	—	—	—	—	0	—	0

Effector-to-target ratios for the specific cell lines were 2.5:1 for clones from subject 68 and 5:1 for clones from subject 63, except 63I9, which was tested at 10:1. Spontaneous lysis was always <30%, except for target cells incubated with peptide 55, which was toxic to target cells, with spontaneous release values ranging from 40–70%. Boxes indicate the regions predicted by the vaccinia truncation construct data to contain the recognized epitope. —, Not tested; NA, data not available due to spontaneous release >30%. The amino acid sequences of RT peptides found to sensitize target cells were IETVPVKLKPGMDGPKVKQWPLTEE (peptide 40), AIFQSSMTKILEPFRKQNPDIVIYQ (peptide 49), NPDIVIYQYMDDLYVGSDLEIGQHR (peptide 50), DLEIGQHRTKIEELRQHLLRWGLTT (peptide 51), PLTEEAELELAENREILKEPVHGVY (peptide 57), and EIQKQGQGQWTYQIYQEPFKNLKTG (peptide 59).

negative (data not shown), indicating that the clones themselves are not infected with HIV-1.

DISCUSSION

These results indicate that multiple epitopes of a given viral gene product can act as immunogens for the cytotoxic T-cell-mediated immune response in HIV-1-infected individuals. For each CTL clone examined, the epitope mapping using vaccinia-HIV-1 truncation constructs correlated precisely with data obtained using synthetic RT peptides to sensitize target cells. None of the viral peptides containing CTL epitopes was recognized by CTL from both subjects studied, suggesting that the immunogenic viral epitopes are different in different individuals. The full spectrum of epitopes recognized by RT-specific CTL is yet to be determined, but it may be large; in this study, CTL restricted by four different HLA class I antigens recognized five different peptide-defined epitopes. The demonstration that more than one immunogenic viral epitope of a given viral gene product can associate with the same HLA class I antigen (A11, in this case) is further evidence for the heterogeneity of the HIV-1-specific CTL response.

These data also indicate that CTL directed at the viral RT should be at least partially cross-reactive. Peptides demonstrated in these studies to contain CTL epitopes were examined for sequence similarity among sequenced HIV-1 isolates. In each case, considerable sequence similarity was present, indicating that CTL specific for these epitopes should cross-react with more than one viral isolate. For example, the predicted amino acid sequence for peptide 40 (amino acids 172–196) is identical for the HIV-1 isolates HXB.2, HB102, HB5, PV22, BRU, RF, MN, SF2, and ELI and differs by only one amino acid for the HIV-1 MAL isolate (31). The least conserved sequence for these peptides containing CTL epitopes was that of peptide 51 (amino acids 359–383), which differed by as many as four amino acids among these sequenced isolates. It will be of interest to determine if the minimal complement of amino acids necessary for recognition by these clones will fit computer-assisted predictions of T-cell epitopes (32, 33).

This establishment of long-term cultures of human virus-specific CTL provides a useful reagent for the elucidation of mechanisms involved in cell-mediated immunity. Detailed mapping of the immunogenic epitopes of HIV-1 using the system described here may identify immunodominant CTL epitopes to direct the rational design of an AIDS vaccine that elicits CTL responses. Whether CTL responses to HIV-1 RT or other HIV-1 proteins will protect from initial infection or retard disease progression remains unknown. If CTL responses are of importance in HIV-1 vaccine development, our data suggest that a subunit vaccine may need to be polyvalent to be immunogenic in individuals of many diverse HLA types. The system described here should help in the development of strategies for both active immunization against HIV-1 as well as for passive cellular immunotherapy in HIV-1-infected individuals.

Table 5. RT-specific lysis by clone 68A62

	% specific cytotoxicity	
Days in culture	VAC/lac	VAC/RT
67	6	71
77	2	66
91	5	75
104	2	74
111	1	77
154	2	98
168	0	66
202	0	66
265	1	67
314	3	54

Target cells are autologous lymphoblasts infected with the control vaccinia virus (VAC/lac) or the RT-expressing vaccinia virus (VAC/RT). Results are shown at an effector-to-target ratio of 2.5:1, in 4-hr chromium release assays.

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