JOURNAL OF VIROLOGY, May 1992, p. 3151–3154
0022-538X/92/053151-04$02.00/0

Vol. 66, No. 5

Mutation of Human Immunodeficiency Virus Type 1 at Amino Acid 585 on gp41 Results in Loss of Killing by CD8$^+$ A24-Restricted Cytotoxic T Lymphocytes

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A human leukocyte antigen A24-restricted CD8$^+$ cytotoxic T-cell clone specific for gp41 of human immunodeficiency virus type 1 was isolated from an infected individual. The epitope was localized to amino acids 584 to 591 (YLKDQQLL, NL43 *env* sequence) of gp41 by using a panel of recombinant vaccinia viruses that contain truncated *env* genes and synthetic peptides. The clone killed autologous B-lymphoblastoid cell lines pulsed with a synthetic peptide reflecting the sequence of the IIIB and MN strains. This clone, however, failed to kill target cells pulsed with the peptides that have a mutation from Lys to Arg or Gln at amino acid 585 which is present in some prototype human immunodeficiency virus type 1 strains, e.g., ADA, JFL, SC, ALA1, BAL1, SF2, VRF, SF33, and WMJ2. This finding that a mutation at amino acid 585 on gp41 results in nonrecognition by human leukocyte antigen A24-restricted CD8$^+$ cytotoxic T lymphocytes suggests that antigenic variation at T-cell epitopes contributes to the failure of immune control of human immunodeficiency virus type 1 infections.

There is a failure of the immune system to eliminate human immunodeficiency type 1 (HIV-1)-infected cells. It is assumed that the HIV-1-specific cytotoxic T-lymphocyte (CTL) responses help to limit virus replication by destroying virus-infected cells in vivo but eventually the infection progresses and immunodeficiency is complicated by numerous infections with opportunistic agents. There have been several reports concerning HIV-1-specific CTL which have been detected directly by using peripheral blood mononuclear cells (PBMC) of HIV-1-infected individuals (5, 9, 11, 12, 15, 16); therefore, there is evidence of ongoing antigenic stimulation by HIV-1-infected cells during HIV-1 infection. To learn more precisely about human T-cell responses to HIV-infected cells, we and others have attempted to clone HIV-1-specific T cells (3, 6, 13, 16). The amount of available information which defines virus-specific T-cell epitopes at the clonal level is limited. For example, only two epitopes restricted by different major histocompatibility complex class II-restricting elements have been defined on HIV-1 gp160 (3, 13). Recently, our laboratory defined in detail the first CD8$^+$ CTL epitope on gp41, amino acids (aa) 768 to 778, which was restricted by human leukocyte antigen (HLA) A3.1 (14a). In addition, we demonstrated that a CD8$^+$ T-cell clone specific for this epitope on gp41 recognized and lysed target cells infected with an HIV-1 strain which shared this sequence, similar to lysis observed by using vaccinia virus/gp160 hybrid virus-infected cells or target cells pulsed with synthetic peptides matching the sequence. Plata et al. used peptide-coated target cells and bronchoalveolar lavage effector cells to demonstrate an HLA A2-restricted epitope at aa 381 to 392 on gp120 (11). Recently, Clerici et al. reported that CD8$^+$ CTL of some HIV-1-infected individuals recognized four synthetic peptides based on the HIV-1 IIIB sequence. These experiments were done in bulk culture, and HLA A2 was identified as a restricting element for all four peptides, and A1 or B8 may also have presented some peptides (1).

In this report, we describe a second CD8$^+$ CTL epitope on gp41 which was localized to aa 584 to 591 of gp41 by using the PBMC of another HIV-1-infected donor. The clone recognized target cells expressing gp41 of the HIV-1 IIIB and MN prototype strains and also killed target cells pulsed with a synthetic peptide reflecting the sequence of the HIV-1 JRCSF strain. This clone failed to recognize target cells pulsed with peptides with a mutation from K to R or Q at aa 585 and thus failed to kill target cells expressing sequences of some prototype HIV-1 strains which have been sequenced, for example, ADA, JFL, SC, ALA1, BAL1, SF2, VRF, SF33, and WMJ2. Therefore, a mutation at aa 585 on gp41 results in nonrecognition by HLA A24-restricted CD8$^+$ CTL.

MATERIALS AND METHODS

Recombinant vaccinia viruses. Recombinant vaccinia viruses that express four different N-terminal overlapping Env proteins of 502, 635, 747, and 851 aa of the BH8 isolate were constructed as previously described (2). HeLa cells infected with these viruses were trypsinized, sonicated, and stored at −80°C before use.

Synthesis of peptides. Synthetic peptides of HIV-1 gp160 (NL43 strain) were constructed as previously described (14a). Some peptides were synthesized by the RaMPS system (Du Pont, Boston, Mass.). The amino acid composition of each peptide was analyzed by using the Waters PICO TAG Amino Acid Analysis System. Envelope protein sequences and numbering of residues are based on the report of Myers et al. (8).

Establishment of an HIV-1 *env*-specific CTL clone. CTL clones were generated as previously described (6). In brief, PBMC of an asymptomatic HIV-1-seropositive subject were seeded at 50 per well in 96-well plates and cultured with CD3-specific monoclonal antibody 12F6 and irradiated allogeneic PBMC from a normal healthy adult as feeder cells in RPMI 1640 medium containing 10% fetal calf serum, antibiotics, and recombinant human interleukin-2 (Cellular Prod-

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ucts, Buffalo, N.Y.). Plates were fed once a week with fresh medium, and 2 weeks later the cells were restimulated with 12F6 and allogeneic feeders. After 4 weeks, the cells were screened for lytic activity on autologous B-lymphoblastoid cell lines (B-LCL) infected with vPE16, which expresses HIV-1 gp160 or the NYCBH strain of vaccinia virus (negative control). T-cell lines demonstrating specific lysis of targets infected with vPE16 were cloned at limiting dilution in the presence of 12F6, recombinant human interleukin-2, and feeder cells.

CTL assay. CTL activity was measured in a standard Cr release assay as previously described (6). In brief, Epstein-Barr virus-transformed B-LCL were infected at 20 PFU per cell with recombinant vaccinia virus containing the entire gene for HIV-1 gp160 or truncated segments of the gene or with vaccinia virus as a negative control for 14 to 15 h. The cells were labeled with Na-chromate and used as target cells. In experiments with peptide-pulsed target cells, the cells were labeled with Na-chromate before pulsing with synthetic peptides for 1 h. Effector cells were added to target cells in triplicate and incubated for 4 h at 37°C. Percent specific lysis was calculated by the formula 100 × [(mean test counts per minute − mean spontaneous counts per minute)/(mean maximal counts per minute − mean spontaneous counts per minute)]. Spontaneous release of target cells was always less than 30%.

Phenotypic analysis of cells. Cells were incubated with a fluorescein isothiocyanate-labeled monoclonal antibody specific for CD3, CD4, CD8, and CD16; washed; and analyzed by fluorescence-activated cell sorter (Beckton Dickinson, San Jose, Calif.).

Nucleotide sequence accession number. The GenBank accession number of HIV NL43, to which we referred for synthesis of the peptides used here, is M19921.

RESULTS

We used methods previously reported by Walker et al. and ourselves (6, 16) to establish long-term T-cell lines directly from the PBMC of an HIV-1-infected donor. Wells were seeded with 50 PBMC per well and supported by irradiated allogeneic feeder cells. Well 137 lysed an autologous B-cell line infected with vaccinia virus/gp160 (IIIB) but did not lyse control target cells infected with vaccinia virus or with vaccinia virus expressing HIV *gag* or *pol* (data not shown). To localize the region of gp160 that was recognized by this CTL line, target cells were infected with vaccinia virus expressing truncated regions of envelope gp160. The results in Table 1 show that the epitope lies between aa 502 and 635 on gp160. In the same experiment, monoclonal antibodies to cross-reactive framework determinants on HLA class I (W6/32) or II (DP, DQ, and DR) were added to the assay. Significant reduction of cytotoxicity was observed only when the monoclonal antibody to HLA class I was used.

T-cell line 137 was tested on target cells pulsed with synthetic peptides based on the sequence of gp41 of HIV-1 IIIB, because line 137 killed target cells infected with vaccinia virus expressing gp160 but not gp120 (Table 1). The results shown in Table 2 indicate that the CTL line recognized target cells that were pulsed with peptide containing aa 564 to 591, but target cells pulsed with peptides representing aa 550 to 574 or 587 to 604 were not recognized.

Parental T-cell line 137 was subcloned at 0.3 and 3.0 cells per well. A number of wells showed growth, and the cells in most wells showed specific killing of autologous B cells infected with vaccinia virus/gp160 or after pulsing with peptide 84. Subclone 137.6 provided a high level of killing of both of these target cells, so it was used for characterization. Clone 137.6 was analyzed by fluorescence-activated cell sorter with monoclonal antibodies to CD3, CD4, and CD8, which revealed it to be a $CD8^+$ clone, since all of the cells present were $CD3^+$, $CD8^+$, and $CD4^-$ (data not shown).

To define the epitope recognized by clone 137.6 in detail, a series of synthetic peptides representing segments contained within aa 564 to 591 were prepared. The results shown in Table 3 indicate that the epitope recognized by 137.6 and another subclone, 137.19, is contained in aa 580 and 591.

We then prepared a series of synthetic peptides with N-terminal truncations from aa 580 to 591. In addition, we synthesized peptides for the region from 580 to 591 to represent mutant virus strains at sites in this region on the basis of published sequence data on HIV-1 strains (8). The results shown in Table 4 indicate that aa 584 to 591 contained the epitope recognized by the $CD8^+$ clone. Lysis of target cells pulsed with peptides representing mutations in the sequences of other prototype strains indicated that a change from K to R or Q at aa 585 resulted in nonrecognition by the clone. A peptide based on the sequence of JRCSF, which has a mutation at 590 from L to M, was recognized. Therefore, certain strains, such as IIIB, MN, and JRCSF, were recognized by this $CD8^+$ CTL clone but a mutation at

TABLE 1. Localization of epitope gp160 with recombinant vaccinia viruses containing truncated HIV-1 *env* genes

Virus	Truncated Env protein (amino acid residues[a]) or MAb[b]	% Specific lysis[c]
Recombinant vaccinia viruses		
vPE16	1–851	94.8
vPE17	1–747	91.8
vPE18	1–635	82.8
vPE8	1–502	0.0
Vaccinia virus		0.6
Recombinant vaccinia virus		
vPE16	Anti-class I	56.4
	Anti-DP	87.5
	Anti-DQ	78.5
	Anti-DR	87.6
	None	94.8

[a] Numbering of amino acid residues is based on the HIV-1 IIIB strain.
[b] MAb, monoclonal antibody.
[c] CTL activity was determined at an effector-target cell ratio of 4.

TABLE 2. Mapping of the epitope using synthetic envelope peptides

Envelope peptide	Amino acid residues	% Specific lysis[a]
80	510–530	−0.6
81	522–546	0.0
82	534–554	−2.7
83	550–573	1.9
84	564–591	33.9
85	587–607	2.5
86	599–625	−0.4
87	616–642	0.3

[a] Autologous B-LCL were pulsed with each peptide at a concentration of 50 μg/ml for 1 h and used for CTL assay at an effector-target cell ratio of 4.

TABLE 3. Epitope mapping using overlapping synthetic peptides

Envelope peptide	Amino acid residues	% Specific lysis[a] by clone: 137.6	137.19
84	564–591	42.3	60.7
84A	555–570	−4.7	−2.7
84B	560–575	−2.5	−1.8
84C	565–580	−1.4	2.4
84D	570–585	0.7	2.2
84E	575–590	−4.2	−3.4
84F	580–595	54.3	43.0
84G	585–600	2.1	−1.1

[a] Autologous B-LCL were pulsed with each peptide (50 μg/ml) and tested for CTL assay at an effector-target cell ratio of 5.

site 585 from K to R or Q resulted in nonrecognition of a number of other prototype strains, for example, ADA, JFL, SC, ALA1, BAL1, SF2, VFR, SF33, and WMJ2.

Experiments were performed to define the HLA-restricting element used by this $CD8^+$ CTL clone. The results shown in Table 5 indicate that allogeneic target cells which share A24 as the common HLA antigen were recognized and lysed by clone 137.6 when they expressed this epitope. Allogeneic target cells which shared another HLA antigen, A1, B8, or B39, were not lysed.

DISCUSSION

Several reports have defined HIV-1-specific T-cell epitopes on *gag* and *pol*, but there is a relative scarcity of reports of $CD8^+$ CTL epitopes on envelope gp160. Earlier, Plata et al. (11) noted that killing of peptide-pulsed target cells by bronchoalveolar effector cells was A2 restricted and localized to the region from aa 381 to 392 on gp120. Recently, we defined an epitope on gp41 localized between 768 and 778 (14a). Clerici et al. (1) recently reported that synthetic peptides representing four sites on gp160 of the IIIB strain were recognized by $CD8^+$ T cells of some HIV-1-seropositive donors. The epitopes were not defined in detail. HLA A2 restriction was reported with the four peptides, but A1 or B8 may also have presented some peptides. Thus, only a few human $CD8^+$ CTL epitopes have been defined on the HIV-1 envelope glycoprotein. The reasons for this discrepancy between the reported numbers of epitopes on HIV-1 *env* and epitopes on *gag* and *pol* are not known; however, it may be due in part to the methods used to identify HIV-specific CTL clones. We and other investigators screen candidate human T-cell clones for HIV-1 specificity by the ability to lyse autologous B cells infected with vaccinia virus expressing genes for the prototype IIIB virus (6, 16). Thus, the only T-cell lines which are positive in screening cytotoxicity assays are those which specifically lyse vaccinia virus expressing HIV-1 IIIB genes. Since the degree of variation of the envelope glycoprotein exceeds that of the Gag, Pol, or other internal proteins (8), it might be expected that multiple mutations in gp160 between (i) the strain(s) of the virus infecting the individual and inducing the $CD8^+$ CTL responses and (ii) the sequence in the region of the IIIB epitope of gp160 are different. It is likely that $CD8^+$ CTL clones that recognize autologous virus-infected cells but not vaccinia virus/gp160 IIIB-infected cells are not detected.

Two reports have described $CD4^+$ CTL epitopes on the envelope glycoprotein, and both reports have involved the use of recombinant baculovirus-expressed gp160, either as an in vitro stimulus of uninfected donor PBMC (13) or after immunization of an HIV-1-seronegative individual with experimental recombinant baculovirus-expressed gp160 vaccine (10). Following administration of the experimental vaccine, a $CD4^+$ HLA DPw4-restricted T-cell clone was derived and mapped to a region which overlaps the epitope on gp41 detected with a $CD8^+$ CTL clone from an infected donor described in this report (3). There have been several reports in murine T-cell systems of specific T-cell determinants on proteins which overlap epitopes that can be presented to CD4 and CD8 T cells (14). We have defined a highly conserved HLA Cw3-restricted $CD8^+$ CTL epitope on HIV-1 p24 aa 140 to 148 (6) and recently reported an HLA DQ-restricted $CD4^+$ CTL epitope to p24 aa 145 to 150 which partly overlaps the $CD8^+$ CTL epitope (6a). In these cases, partial overlap of epitopes was observed but fine epitope mapping indicated differential recognition of sequences by $CD4^+$ and $CD8^+$ T cells. These observations indicate that certain regions of viral proteins, and other antigens, contain overlapping regions of peptides that are immunogenic for both CD4 and $CD8^+$ T-cell responses. This has practical potential, since the ability to interact with both class I and

TABLE 4. Fine epitope mapping using truncated synthetic peptides

Envelope peptide	Amino acid sequence	Amino acid residues	% Specific lysis[a] by clone 137.6
84F	AVERYLKDQQLL	580–591	42.2
84F1	VERYLKDQQLL	581–591	54.3
84F2	ERYLKDQQLL	582–591	43.3
84F3	RYLKDQQLL	583–591	59.9
84F4	YLKDQQLL	584–591	48.6
84F5	LKDQQLL	585–591	−8.8
8F46	YLKDQQL	584–590	2.2
I[b]	YLRDQQLL	584–591	−5.8
II[b]	YLQDQQLL	584–591	−3.7
III[b]	YLKDQQLM	584–591	37.7

[a] Autologous B-LCL were pulsed with each peptide (50 μg/ml) and tested for CTL assay at an effector-target cell ratio of 5.

[b] Sequence data are from the compilation of Myers et al. (8).

TABLE 5. HLA restriction of clone 137.6

Target B-LCL	HLA antigens with the following phenotype: Class I	Class II	% Specific lysis[a]
11	A1, A24, B8, B39, Cw−	DR3, DR6, DQw1, DQw2	59.8
CG	A1, A2, B8, B17, Cw6, Cw7	NT[b]	−2.7
MTT	A2, A24, B49, Bw55, Cw3	NT	25.2
MHN	A24, A30, B13, Bw55, Cw4, Cw6	NT	54.0
15	A2, A24, B35, B49, Cw4, Cw8	DR2, DR5, DQw1, DQw3	55.7
JC	A3, A24, B35, Cw4	DR2, DR4, DQw1, DQw3	35.3
BI	A26, A29, B35, B39, Cw4	DR5, DQw1, DQw3	6.1

[a] Autologous and allogeneic B-LCL which share an HLA antigen(s) with the donor were infected with vPE18 recombinant vaccinia virus and used for CTL assay at an effector-target cell ratio of 5.

[b] NT, not tested.

class II major histocompatibility complex antigens suggests that T cells of a larger number of individuals would recognize such epitopes on virus-infected cells or after immunization with a vaccine which contains these peptide regions.

The importance of $CD8^+$ CTL responses in HIV-1 infections remains to be determined. In the absence of an appropriate animal model, it has not been possible to test directly the effect of adoptive transfer of CTL clones specific for HIV-1. Studies with the Hu/SCID mouse model which should be of interest are being attempted. One of the major questions concerning the biology of HIV-1-specific CTL responses is their failure to eliminate HIV-1-infected cells and resolve HIV-1 infections despite evidence that virus-specific $CD8^+$ CTL can cure T-cell-deficient mice with other virus infections (7), including persistent established lymphocytic choriomeningitis virus infection in nude mice (4). The ability of HIV-1 and other lentiviruses to insert provirus into host cell DNA (17), antigenic variation at T-cell epitopes such as those described in this report, and the impact of HIV-1 infections and immune responses to HIV-1-infected immunocompetent cells may contribute to the failure of human immune systems to resolve HIV-1 infections.

ACKNOWLEDGMENTS

We are grateful to Patricia Earl and Bernard Moss for providing the recombinant vaccinia virus containing HIV-1 genes, Thomas Fuerst for providing some of the synthetic peptides to HIV-1 gp160, and Anita Leporati for preparing recombinant vaccinia virus stocks.

This work was supported by NIH research grants RO1-AI24750 and NIH-T32-AI 07272.

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