Specific lysis of human immunodeficiency virus type 1-infected cells by a HLA-A3.1-restricted CD8⁺ cytotoxic T-lymphocyte clone that recognizes a conserved peptide sequence within the gp41 subunit of the envelope protein

(AIDS/T-cell epitope)

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ABSTRACT   A HLA-A3.1-restricted CD8⁺ cytotoxic T-cell clone, E7.20, that lyses cells infected with human immunodeficiency virus type 1 was isolated from an infected individual. The epitope was localized to amino acids 768–778 (RLRDLLLIVTR, NL43 env sequence) of the cytoplasmic domain of gp41 by successive use of a panel of recombinant vaccinia viruses that express truncated env genes and synthetic peptides. The epitope is conserved on 7 (NL43, BRU, HXB2, BRVA, SC, JH3, and JFL) of 13 human immunodeficiency virus type 1 isolates from North America. Synthetic peptides of this region of strains RF and CDC4 are also recognized by E7.20 despite a nonconservative Thr → Val or Thr → Ala change at amino acid 777; however, an MN peptide, which has four amino acid substitutions, was not reactive. The epitope recognized by E7.20 has a predicted hydrophobic α-helical structure, with three contiguous Leu residues followed by Ile and Val at amino acids 772–776. Cytotoxicity was restricted by HLA-A3.1 using allogeneic target cells that shared HLA class I antigens with the donor and an HLA-A and -B negative human plasma cell line transfected with the HLA-A3.1 gene. The transfected cells were infectable by human immunodeficiency virus type 1 strains IIIB and MN but only the former virus sensitized them to killing by E7.20. The ability of E7.20 to specifically lyse a human lymphocyte line infected with a human immunodeficiency virus type 1 strain carrying the conserved epitope is consistent with an important role for cytotoxic T cells in controlling infection.

T-cell responses play an important role in the host defense against many viruses (1–4). Usually, cytotoxic T lymphocytes (CTLs) are present during the acute and convalescent stages of virus infections. Human immunodeficiency virus (HIV) appears to be a notable exception in that CTLs have been isolated from the blood during most, if not all, stages of disease (5–12). Although CTLs are evidently unable to eliminate HIV, it is generally assumed that the course of disease would be more rapid in their absence. CTL stimulation is therefore considered to be desirable in developing vaccine and immunotherapy strategies. T-cell epitopes and their restriction elements for HIV type 1 (HIV-1) gag (9), pol (13), env (14–17), and nef (18, 19) have been identified by their reactivity to T-cell clones or in bulk peripheral blood mononuclear cell (PBMC) cultures of humans and mice (20). However, information that is available concerning the precise epitopes and their HLA restriction as defined by estab-

lished CTL clones is still very limited. For example, concerning well-characterized human CTL epitopes on gp160, only one epitope on gp120 recognized by CD8 CTLs has been reported by Plata and coworkers (6, 14), and one epitope each on gp120 and gp41 recognized by CD4 CTL clones has been reported by Siliciano et al. (15) and Hammond et al. (16). The definition of such T-cell epitopes and their HLA restriction will contribute to strategies for HIV vaccine development and to our understanding of antigenic variation at the T-cell level.

Another limitation of previous reports is that CD8 CTL specificity was demonstrated only using recombinant vaccinia viruses or peptide-treated target cells. In the present study we have defined a conserved epitope within the gp41 subunit of the envelope protein of HIV-1 and demonstrated that a cloned HLA-A3.1-restricted CTL clone was capable of killing cells infected with HIV-1 in vitro.

MATERIALS AND METHODS

Recombinant Vaccinia Viruses. Recombinant vaccinia viruses that express seven different N-terminal overlapping envelope proteins of 204, 287, 393, 502, 635, 747, and 851 amino acids of the BH8 isolate were constructed as described (17). HeLa cells infected with these viruses were trypsinized, sonicated, and stored at −80°C before use.

Synthesis of Peptides. Synthetic peptides with ≈25 amino acids of HIV-1 gp160 (NL43 strain) that overlapped each other by 12 or 13 residues were constructed using an Applied Biosystems model 430A peptide synthesizer. The peptide was purified using a Vydac C-4 reversed-phase column and Beckman system gold HPLC to >95% homogeneity. Some peptides were synthesized by the RaMPS system (DuPont). The amino acid composition of each peptide was analyzed 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. (21).

Cell Lines. Epstein–Barr virus-transformed B-lymphoblastic cell lines (B-LCLs) were established by infecting the PBMCs of a HIV-1-infected individual with B95-8 supernatant. The human plasma cell line HMy.C1R was transfected by calcium phosphate precipitation with a plasmid clone containing the HLA-A3.1 gene (22). The HMy.C1R cell line does not express HLA-A and -B molecules as determined by

Abbreviations: CTL, cytotoxic T lymphocyte; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; B-LCL, B-lymphoblastic cell line; MHC, major histocompatibility complex; E/T, effector-to-target.
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HLA typing. The transfected cells, HMy.C1R-A3.1, were assayed for surface expression of A3 molecules by indirect immunofluorescence using the HLA-A3-specific monoclonal antibody GAP.A3 (23).

Establishment of a HIV-1 env-Specific CTL Clone. PBMCs were obtained by Ficoll/Hypaque density gradient centrifugation of blood from an asymptomatic HIV-1 seropositive subject (Walter Reed stage 2). The circulating lymphocytes of this patient showed cytolytic activity for autologous B-LCL cells infected with recombinant vaccinia virus containing a HIV-1 *gag* or *env* gene (24). PBMCs were seeded at 50 cells per well in 96-well plates and cultured with CD3-specific monoclonal antibody 12F6 (13) and irradiated allogeneic PBMCs from a normal healthy adult as feeder cells in RPMI 1640 medium containing 10% fetal calf serum, antibiotics, and 10% T-cell growth factor (TCGF) (Cellular Products). Plates were fed once a week with fresh medium. After 4 weeks, the cells were screened for lytic activity of autologous B-LCL cells 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 phytohemagglutinin P (5 μg/ml; GIBCO) and TCGF.

CTL Assay. CTL activity was measured in a standard Cr release assay. In brief, B-LCL cells were infected with 5 plaque-forming units per cell of 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 14–15 hr. The cells were labeled with sodium chromate and were used as target cells. In experiments with peptide-pulsed target cells, the cells were labeled with sodium chromate before pulsing with synthetic peptides for 1 hr. Effector cells were added to target cells in triplicate and incubated for 4 hr at 37°C. For HIV-1-infected target cells, HMy.C1R-A3.1 cells were infected with HIV-1 IIIB or MN strain at a multiplicity of infection of 1 or mock-infected and cultured for 7 days in RPMI 1640 medium containing 10% fetal calf serum. The cells were washed, labeled with sodium chromate, and tested for CTL activity. The expression of HIV-1 antigens was detected by fluorescence-activated cell sorter analysis using anti-HIV-1 gag p24 monoclonal antibody (SmithKline Beecham). Percent specific lysis was calculated by the formula 100 × [(mean test cpm − mean spontaneous cpm)/(mean maximal cpm − mean spontaneous cpm)]. Spontaneous release of the target cells was always <30%.

Phenotypic Analysis of Cells. Cells were incubated with fluorescein isothiocyanate-labeled monoclonal antibody specific for CD3, CD4, CD8, and CD16, washed, and analyzed by fluorescence-activated cell sorting (Beckton Dickinson).

RESULTS

Generation of a HIV-1 env-Specific CTL Clone. PBMCs from an asymptomatic HIV-1 seropositive subject were cul-

tured with CD3-specific monoclonal antibody and allogeneic feeder cells. The proliferating cells were screened for CTL activity on autologous B-LCL cells infected with vPE16, a recombinant vaccinia virus that expresses the entire HIV-1 gp160 gene. A T-cell line (E7) possessed HIV-1-specific CTL activity—i.e., it lysed targets expressing gp160 (35.2% specific lysis) but did not lyse control vaccinia virus-infected targets (0%) or a natural killer sensitive cell line, K562 cells (1.7%). E7 was cloned by limiting dilution at 10 cells per well. Four weeks later 23 of 192 wells had proliferating cells and those in all 23 wells exhibited varying degrees of *env*-specific lytic activity. Based on a Poisson distribution, these cells are considered to be clones (25). The clone (E7.20) that exhibited the highest level of lytic ability was used for further studies. Phenotypic analysis revealed E7.20 to be $CD3^+$, $CD8^+$, $CD4^-$, and $CD16^-$ (Leu11) (data not shown).

Epitope Mapping of E7.20 Using Recombinant Vaccinia Virus Containing Truncated env Genes. The epitope recognized by E7.20 was examined with recombinant vaccinia viruses expressing the envelope protein with sequentially greater carboxyl-terminal deletions. As shown in Fig. 1, E7.20 was highly cytolytic to autologous B-LCL target cells infected with vPE16, which express the entire gp160 sequence. It also lysed vPE17-infected target cells to a lower level. This result indicates that one or more epitopes recognized by E7.20 are in gp41 between amino acids 636 and 851.

Fine Epitope Mapping of E7.20 with Synthetic Peptides. To define the epitope(s) recognized by E7.20, autologous B-LCL cells were pulsed with overlapping 25-amino acid synthetic peptides spanning amino acids 630–854 and tested for cytotoxicity. As shown in Fig. 2, only envelope peptide 40 (amino acids 760–784) located in the central portion of the cytoplasmic domain of gp41 was recognized by E7.20. The possibility that a second epitope exists, between amino acids 635 and 747, but was not detected with the panel of peptides has not been ruled out. The two peptides adjacent to envelope peptide 40 (envelope peptides 39 and 41) were not recognized, suggesting that at least part of the epitope lies between envelope peptides 39 and 41—i.e., the region including amino acid 772. To define the epitope more precisely, progressively smaller peptides in the junctional portion were synthesized and tested at 20 μM in CTL assays (Table 1). First, peptide 40A was tested and found to sensitize target cells. Peptides lacking two or three residues at the amino-terminal end of peptide 40A (40B and 40C) were reactive, but additional amino-terminal truncation (peptide 40D) resulted in a decrease in activity, and the nonapeptide 40E did not sensitize target cells. On the other hand, peptide 40F, lacking Arg at the carboxyl-terminal end of peptide 40A, also drastically lost the ability to be recognized by E7.20. To confirm the decrease of cytolytic activity with peptide 40D, a dose–response experiment was done (Fig. 3). Target cells pulsed

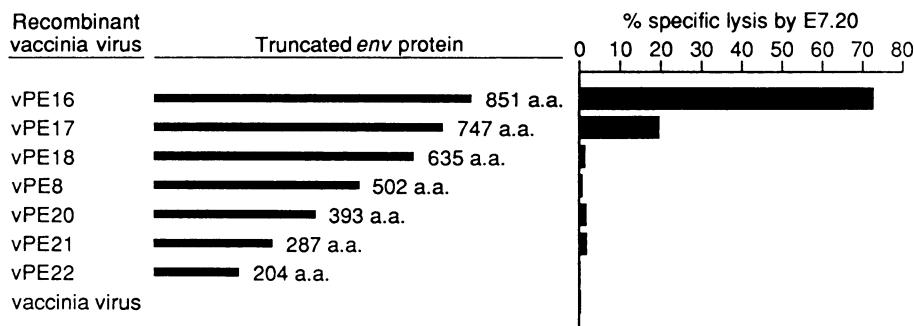| Recombinant vaccinia virus	Truncated *env* protein	% specific lysis by E7.20
vPE16	851 a.a.	
vPE17	747 a.a.	
vPE18	635 a.a.	
vPE8	502 a.a.	
vPE20	393 a.a.	
vPE21	287 a.a.	
vPE22	204 a.a.	
vaccinia virus		

Fig. 1. Mapping of the E7.20 epitope using recombinant vaccinia viruses containing truncated HIV-1 *env* genes. The total number of amino acids (a.a.) of the HIV-1 *env* encoded by each recombinant vaccinia virus is indicated. Autologous B-LCL cells were infected with the indicated recombinant vaccinia virus (multiplicity of infection = 5) or with vaccinia virus and then tested in a CTL assay at an effector-to-target (E/T) ratio of 5.
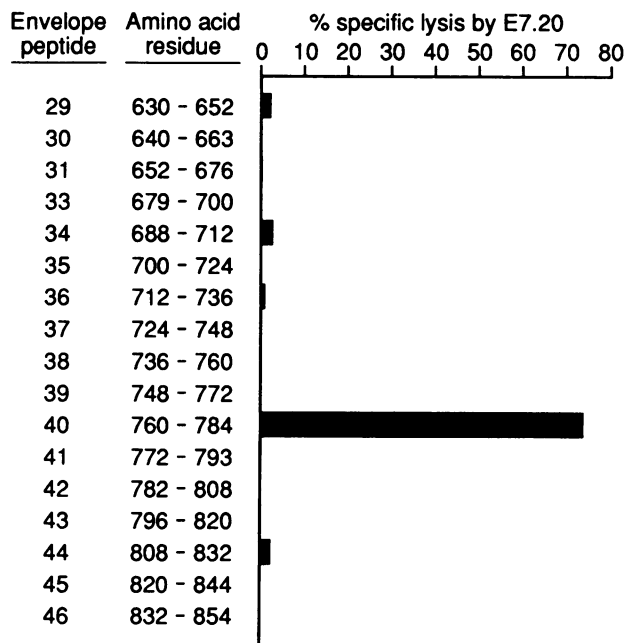
FIG. 2.   Mapping of the E7.20 epitope using overlapping synthetic peptides. Autologous B-LCL cells were pulsed with the 25-amino acid peptides in gp41 (NL43; ref. 21) and then tested in a CTL assay at an E/T ratio of 5.

FIG. 3.   Dose–response CTL assay with synthetic peptides. Autologous B-LCL cells (5 × 10^5) were incubated with the indicated concentration of peptide 40C (●) or 40D (○) for 1 hr before ^{51}Cr labeling and tested in a CTL assay at an E/T ratio of 5.

with peptide 40D were lysed to a low level even at 100 μM, whereas target cells pulsed with peptide 40C were killed at 1 μM. These results indicate that the core epitope recognized by E7.20 lies within the 11 residues of amino acids 768–778.

Major Histocompatibility Complex (MHC) Restriction of the CTL Clone. To define the MHC restriction element needed for cytotoxic activity of E7.20, allogeneic B-LCL cells that shared a HLA allele with the donor were infected with vPE16 expressing the whole envelope protein and tested as targets. The HLA type of the donor from whom E7.20 was derived was A3, A25, B18, Bw62, Cw3, DR4, DRw10, DQw1, DQw3, DRw53. As shown in Fig. 4A, allogeneic cells that shared HLA-A3 with E7.20 were lysed as well as autologous target cells in an antigen-specific and MHC class I-restricted manner. Allogeneic cells that shared B18, Bw62, or Cw3 alleles were not lysed by E7.20. HLA-A3 restriction of E7.20 was further analyzed using a HLA-A and -B negative human plasma cell line, HMy.C1R, transfected with the HLA-A3.1 gene (Fig. 4B). E7.20 lysed the HLA-A3.1 transfectant in a MHC class I-restricted manner when pulsed with envelope peptide 40, but it did not lyse the peptide-pulsed HMy.C1R control targets.

HIV Sequence Variability in the E7.20 Epitope. The epitope recognized by E7.20 lies in a region of gp41 that is relatively conserved among various HIV-1 isolates from North Amer-
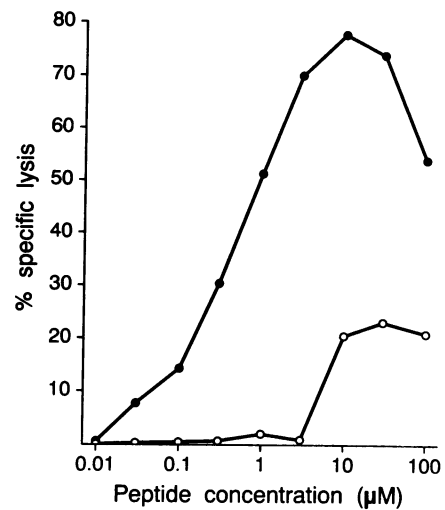
ica, Europe, and Africa (Table 2). Seven isolates from North America have identical sequences in this region. Five amino acids were completely conserved among all isolates and changes were most often observed at residues 777, 776, and 773. To determine whether peptides based on the sequences in this region of other strains could be recognized by E7.20, corresponding synthetic peptides were constructed and tested in a CTL assay. RF and CDC4 peptides sensitized target cells, although they have a nonconservative Thr → Val or Thr → Ala change at residue 777, respectively. Cells incubated with the MAL peptide, which has a conservative Val → Ala change at residue 776, were lysed to a lesser extent. Two Ala substitutions at residues 776 and 777 that appeared in SF2 and MN strains resulted in complete loss in activity.

CTL Activity of E7.20 Against HIV-1-Infected Cells. To determine whether E7.20 could kill HIV-1-infected target cells, HLA-A3.1 transfectant cells (HMy.C1R-A3.1) were infected with the IIIB or MN strain and tested in a CTL assay. As shown in Fig. 5, HMy.C1R-A3.1 cells were susceptible to HIV-1 infection: HIV-1 antigen, p24, was detected in approximately half of the cells infected with either the IIIB or MN strain. E7.20 caused significant cytolysis of the IIIB-infected cells, whereas MN-infected target cells were not lysed. This difference was consistent with the specificity of E7.20 for IIIB and MN peptides (Table 2).

DISCUSSION

In the present study we demonstrated the existence of a relatively conserved epitope on HIV-1 gp41 that is recog-

Table 1.   Fine epitope mapping using progressively smaller peptides

Envelope peptide	Amino acid sequence	Amino acid residues	% specific lysis by E7.20
40	SLCLFSYHRLRDLLLIVTRIVELLG	760–784	69.3
40A	SYHRLRDLLLIVTR	765–778	72.8
40B	HRLRDLLLIVTR	767–778	64.2
40C	RLRDLLLIVTR	768–778	71.2
40D	LRDLLLIVTR	769–778	23.1
40E	RDLLLIVTR	770–778	3.6
40F	RLRDLLLIVT	768–777	3.5
40G	RLRDLLLIV	769–777	0.9

Autologous B-LCL cells were pulsed with each peptide (20 μM) and tested for CTL activity by E7.20 at an E/T ratio of 5.
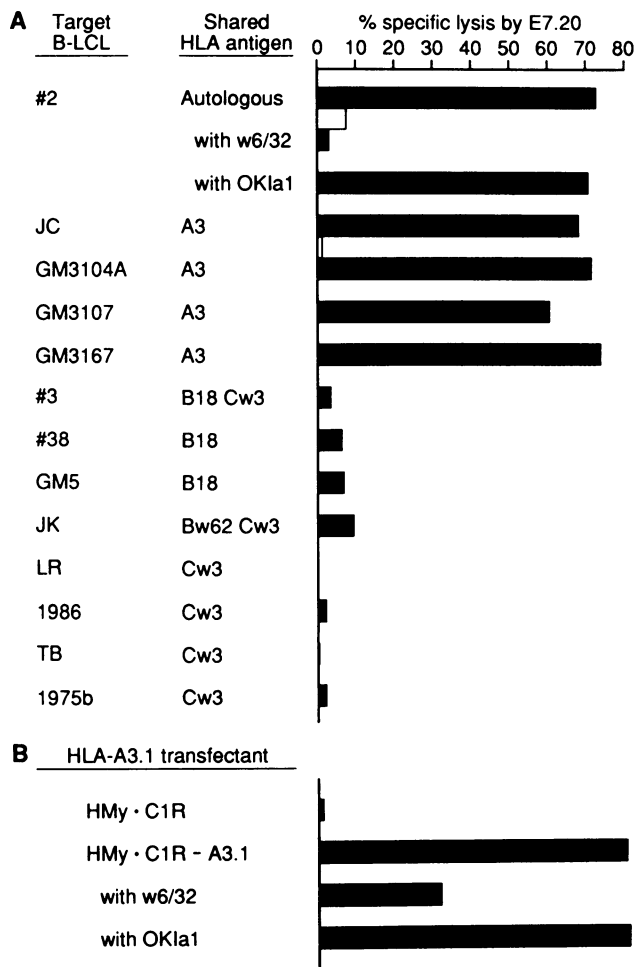
FIG. 4. HLA restriction of E7.20. (*A*) Autologous and allogeneic B-LCL cells that share a HLA antigen(s) with the donor were infected with vPE16 recombinant vaccinia virus (solid bar) containing the entire *env* gene or vaccinia virus alone (open bar) and tested for cytotoxicity by E7.20. HLA typing of allogeneic PBMCs was performed in a standard microcytotoxicity test. Monoclonal antibody W6/32 (anti-class I) and OKIa1 (anti-DR) were used in a blocking experiment of CTL activity in the autologous system. (*B*) Human plasma cell line HMy.C1R and HMy.C1R transfected with HLA-A3.1 gene was pulsed with envelope peptide 40 and used in a CTL assay.

nized by a HLA-A3.1-restricted CD8^{+} CTL clone isolated from a HIV-1-infected individual. Concerning well-characterized human T-cell epitopes on gp160, Plata *et al.* (6, 14) reported a conserved epitope on gp120 (amino acids 381–392) recognized by HLA-A2-restricted CD8^{+} CTLs freshly isolated from broncho-alveolar lavage of an HIV-

Table 2. Amino acid sequence variability in the epitope recognized by E7.20

HIV-1 isolate	Origin*	Amino acid sequence (768–778)	% specific lysis[†]
NL43, BRU, HXB2, BRVA, SC, JH3, JFL	NA	RLRDLLLIVTR	71.2
RF	NA	————————V–	75.8
CDC4	NA	————————A–	60.0
MAL	Z	———————A—	19.3
SF2	NA	———————AA–	5.2
MN	NA	HH———————AA–	4.7
Z2	Z	——————I———AA–	4.4
OVI	GA	——————I————A–	NT
JY1	Z	——————I——A—	NT
WMJ2	NA	————————K–	NT
HAN	G	————————AK–	NT
ELI	Z	——————I——AV–	NT
NDK	Z	—————SI———AA–	NT
Z321	Z	—————CA———AA–	NT

For sequence data refer to Myers *et al.* (21). NT, not tested.
*NA, North America; Z, Zaire; G, Germany; GA, Gabon.
[†]Autologous B-LCL cells were pulsed with each peptide (20 μM) and used for a CTL assay at an E/T ratio of 5.

infected patient. Siliciano *et al.* (15) reported a DR4-restricted CD4^{+} CTL epitope on gp120 (amino acids 410–429), and Hammond *et al.* (16) recently reported a DPw4.2-restricted CD4^{+} CTL epitope on gp41 (amino acids 577–595) in subjects who had been immunized with recombinant gp160. The E7.20 epitope presented here is, therefore, another epitope found on HIV-1 gp41 that is recognized by a CD8^{+} clone in association with HLA-A3.1. This clone was derived directly from the PBMCs and was not stimulated with HIV-1 antigen *in vitro* before it was characterized; therefore, we assume that the infecting virus, which has not yet been defined, induced this CD8^{+} CTL clone.

The region of gp41 (amino acids 768–778) that is recognized by clone E7.20 is conserved on a number of HIV-1 isolates from North America (Table 2; ref. 21). In seven tested strains (BRU, NL43, HXB2, BRVA, SC, JH3, and JFL) the sequence is entirely conserved. A peptide based on the RF sequence of this region is also recognized by E7.20 despite a nonconservative Thr → Val change at amino acid 777. However, the MN peptide, which has four amino acid substitutions, was not reactive.

The structure of the epitope recognized by clone E7.20 is very hydrophobic, with three contiguous Leu residues followed by Ile and Val at amino acids 772–776. Apparently this hydrophobic region is structurally important because the residue LLLI is highly conserved among HIV-1 isolates
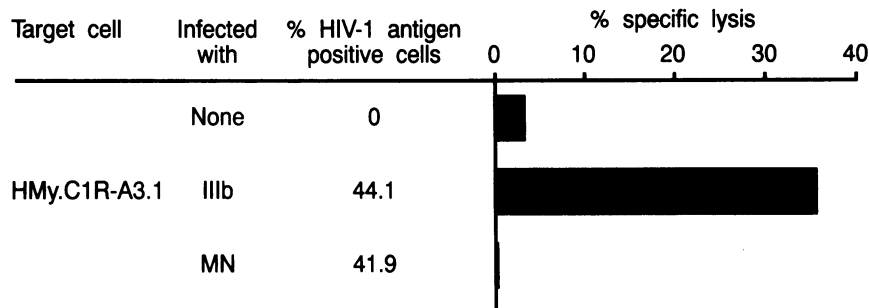

FIG. 5. CTL activity of E7.20 against HIV-1-infected cells. HMy.C1R-A3.1 cells were infected with the HIV-1 IIIB or the MN strain at a multiplicity of infection of 1. The cells were cultured for 7 days and tested for CTL activity by E7.20 at an E/T ratio of 5. The expression of HIV-1 antigens was detected by fluorescence-activated cell sorter analysis using anti-HIV-1 gag p24 monoclonal antibody.

(Table 2). The sequence that is recognized by clone E7.20 does not fit any of three commonly used algorithms for predicting T-cell epitopes (26–28).

When the sequence of E7.20 epitope is compared with that of an epitope in the HIV-1 nef protein (QVPLRPMTYK) that is recognized by CD8$^+$ CTLs in association with the HLA-A3.1 molecule (18), both epitopes have two contiguous Leu-Arg residues as a consensus sequence. This consensus sequence might be important for binding to the HLA-A3.1 molecule.

Cytotoxicity by E7.20 was restricted by the HLA-A3.1 molecule. The frequency of HLA-A3 is 24% and 14% in Caucasian and Black populations, respectively (29). Therefore, a considerable portion of the population would be expected to respond to the E7.20 epitope. However, from the point of view of peptide vaccines, other conserved HIV-1 epitopes that are presented by other HLA molecules should also be taken into consideration.

The persistence of circulating CTLs in HIV-infected individuals presumably reflects continuous HIV replication. Whether these CTLs are important in host defense or are responsible for some pathology is not yet known. We know of no previous published report that describes the killing of HIV-infected cells by a CD8$^+$ CTL clone. Some viruses are known to suppress presentation of antigens in association with MHC class I molecules (30, 31). Whether HIV-1 has this potential is not known. It was therefore of interest to demonstrate the lysis of HIV-1-infected target cells by CD8$^+$ clone E7.20, which suggests that the circulating CTLs can play an important role in eliminating HIV-1-infected cells *in vivo*. The fact that E7.20 lysed IIIB-infected but not MN-infected HLA-A3.1 transfected cells, even though a similar percentage of cells was infected with the two virus strains, was consistent with the specificity documented with peptides.

The finding that the BRU- and the RF-type peptides were recognized by E7.20 also indicates that the donor from whom E7.20 was established was likely infected with a BRU- or RF-type strain rather than an MN-like strain. The further isolation and characterization of HIV-1-specific T-cell clones from infected individuals should help to define the roles of these CTLs in infected persons and the impact of these CTLs on the evolution of HIV-1 strains and to understand interactions between the HIV-1-infected cells and CTLs.

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